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RESISTANCE TO GAMMA-HEXACHLOROCYCLOHEXANE IN MUSCA DOMESTICA L.

by

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I. INTRODUCTION

The increasing influence of mankind on nature for the maintenance of his food supplies and health has brought about considerable changes in the conditions of life of many organisms. Some species succeed in adapting themselves to the new circumstances, undergoing changes that enable them to retain their normal abundance. These changes can be of different kinds. A well-known example in entomology is the occurrence of resistant strains after a period of successful chemical control. This alteration in the environment is very simple, as it consists only of the introduction of a single compound. The development of resistance to poisons is therefore an ideal subject for the study of the process of adaptation. The control of insects can be looked upon as an enormous selection experiment, the scale of which largely compensates for the fact that its conditions are not always too well known. For that reason the resistant populations developed by the use of insecticides in the field are an interesting material for the study of genetical and physiological changes that have occurred.

Research in this field is not only of purely scientific interest, it is also of economic importance. In order to be successful in the control of insect pests we clearly are in need of a better insight into the problems of resistance. The prevention of the development of resistance and the control of insect populations that are already resistant have to be studied. These resistant populations offer a suitable subject for research on these problems.

The development of resistance resulting from chemical control in entomology has already been known for a long time. The outstanding examples, however, have more recently appeared as a result of the use of the chlorinated hydrocarbons, such as DDT and γ-HCH. The most striking case is the rapid development of a very high degree of resistance in the housefly, first to DDT, later to a whole series of other toxicants. As the housefly is at the same time a very suitable experimental animal, most of the investigations concerning resistance have been made with that insect. Work has mainly been done on DDT resistance because it was the first to appear and the technical possibilities were comparatively favourable. Although this resistance is by no means completely explained, many interesting results have so far been obtained.

Resistance to other substances has hardly been looked into. Yet, more information on the resistance to other insecticides in *Musca* would be very desirable. On the one hand, the easy development of resistance to a whole group of chlorinated hydrocarbons points to the involvement of some common factor. On the other, there is strong evidence that the mechanisms by which resistance is brought about are not the same for

all members of this group. As in this laboratory research on the mode of action of γ-HCH was being carried out, it was attractive to study the physiological basis of resistance to this insecticide. This became attainable when it proved possible to use a chemical estimation method of γ-HCH in our physiological research.

A detailed discussion of the literature on the resistance to DDT, especially in *Musca*, will be given as these studies have much in common with our own.

II. DISCUSSION OF THE LITERATURE

There are a number of reviews dealing with resistance in insects (Babers, 1949; Babers and Pratt, 1951; Ebbinge Wubben and Kuiper, 1947; Hess, 1952, 1953; Metcalf, 1955b), in addition there are some books and papers on the subject (Babers, 1953; Busvine, 1954b; Chadwick, 1955; Metcalf, 1955a). We will therefore confine ourselves to some general remarks, followed by a more detailed discussion of the DDT resistance in the housefly, which, up to now has rereived the most attention. The scarce literature on the physiology of resistance to γ-HCH will be dealt with in chapter VIII after the expecimental results have been presented.

GENERAL

Resistant populations arise by selection from susceptible ones. A direct decrease in individual susceptibility as a result of treatment with sublethal doses does not occur, as was found in *Periplaneta* (Chang and Crowell, 1953), *Musca* (Harrison, 1952) and *Drosophila* (Tattersfield and Kerridge, 1955). This would indeed have been of little importance in insects with their short life cycles.

As is to be expected resistance shows itself first in places where intense control programs are carried out. Whether selection will alter a population depends of course on the occurrence of genes that are responsible for the resistant characters. The rate at which alterations are brought about can vary to a large extent and probably is mainly conditioned by the nature and frequency of these genes. There are, for instance, insects that have developed no resistance at all in spite of intense control, whereas some species of mosquitos showed the first cases after some 5 years, and in *Musca* resistance was found already after a period of 1 or 2 years.

These cases are examples of "acquired resistance". It is important that in this case still susceptible strains of the same species exist. This

offers the opportunity of comparing physiological processes in both strains, so that their importance can be better evaluated.

There are also species that show a "natural resistance". These species have a much lower susceptibility to certain insecticides, as compared with the large majority of insects (STERNBURG and KEARNS, 1952; PRADHAM *et al.*, 1952).

Perhaps the natural resistant species may be regarded as the extreme in the series mentioned above: here the resistant population does predominate without any selection. According to this idea the difference between acquired and natural resistance is not essential; it may, therefore, be of importance to compare the properties that cause the natural resistance with those responsible for the acquired resistance.

As for a list of the insects that have developed resistance and the insecticides involved, we may refer to the reviews already mentioned.

Among the many insects that have acquired resistance the case of *Musca* is particular in three respects: the large number of insecticides to which resistance has developed, its high degree and the short time needed for its development. It should be pointed out, however, that *Musca* has not developed resistance to all insecticides with which it has been selected. Till now no resistance has been developed, for instance, to the organic phosphate insecticides, whereas mites and aphids readily gave rise to strains resistant to these substances (WIESMANN, 1954). The causes of these specific differences are completely unknown.

In the species that are able to develop resistance the susceptible population is the normally occurring form. Therefore it can be concluded that this population is better adapted to the natural circumstances, for otherwise the resistant population must be expected to be the predominating one (SMITH, 1941).

Nevertheless many strains are capable of maintaining their resistance for a considerable period without any selection. As these populations can hardly be homozygous for the resistant genes it follows that the difference in viability between the resistant and the normal strain cannot be large. It is an amazing fact that the properties responsible for the difference in susceptibility can vary to such an extent without seriously affecting the normal functions of life.

RESISTANCE IN MUSCA

Two different problems of theoretical interest may be distinguished:

a. a genetical problem, of importance for our insight into the development and the heredity of resistance.

b. a physiological problem, dealing with the properties upon which the differences between the susceptible and resistant strains depend.

Although our own work is confined to the second problem, the genetical literature will also be discussed here, because it may enlarge our insight into the physiological problems.

A. Genetical data

The research on the genetical basis of resistance in *Musca* is hampered by the fact that of this insect but very few genetical data are available. MILANI (1954) has started research in this field. He found a high frequency of recessive mutants carried in the heterozygous state and a rapid decline of fertility upon inbreeding. He concludes from these facts that in *Musca* a very high genetic heterogeneity is maintained resulting in a great adaptability. Only by extending the fundamental research the possibilities will be given for a more thorough investigation of the genetical basis of resistance.

The research on the genetical aspects of resistance in *Musca* has been concentrated on the questions whether there are one or more genes involved and whether there is some connexion between the resistance to a number of different toxicants.

Some workers (BRUCE and DECKER, 1950; NORTON, 1953) have tried to estimate the number of genes involved. Using mass crosses of susceptible and resistant strains they estimated the dose-mortality curves of these strains and their filial generations. The former authors crossed a susceptible and a DDT-resistant strain and obtained filial generations with an intermediate LD 50 and a variance greater than that of the parent strains. From this fact they concluded an influence of multiple factors. As has already been remarked by BARBESGAARD and KEIDING (1955) this can hardly be concluded from these experiments.

A more suitable technique is to study the individual degree of resistance of the offspring of single pairs. This can be done by measuring the time after which knock-down or death is brought about by a certain dosage of the poison. This technique was used by HARRISON (1951, 1953) MAELZER and KIRK (1953) and BARBESGAARD and KEIDING (1955).

There are two kinds of DDT-resistant strains, one is resistant to the lethal effect but not to knock-down, the other which is more commonly found is resistant to both. It follows that resistance to kill can be independent of resistance to knock-down. When in crosses the susceptibility to knock-down was studied the F_1 appeared to be susceptible and the F_2 was segregated in 75 % susceptible to 25 % resistant (HARRISON, 1951, 1953). Therefore the resistance to knock-down is controlled by one recessive gene. When the resistance to the lethal action of DDT was studied, however, an intermediate F_1 was found and a F_2 with a large variance, indicating multiple-gene inheritance.

BARBESGAARD and KEIDING (1955) concluded that in one of their strains a single recessive gene controls resistance to DDT, whereas in an other strain probably more genes were involved. In these strains resistance to kill and resistance to knock-down go hand in hand.

MAELZER and KIRK (1953) crossed a susceptible with a multi-resistant strain. The latter was proved to be heterogeneous, about half of the flies had a much higher resistance than the others. In the part with the lower resistance multiple-gene inheritance seemed to be involved, whereas in the strongly resistant individuals moreover a dominant factor is present in the heterozygous state that has a big influence on the degree of resistance. The flies cannot attain homozygosity for this factor, as it seriously diminishes their viability.

From the work of BARBESGAARD and KEIDING (1955) and BUSVINE and KHAN (1955) it is very probable that resistance to γ-HCH depends on multiple genes. NEWMAN (1953) had already pointed out that this resistance could not depend on a simple mechanism: inbred strains obtained from resistant parents gave after two to three generations among their offspring some susceptible individuals.

BRUCE and DECKER (1950) found the interesting fact that resistance to a certain insecticide arose much faster when the strain was already resistant to another toxicant. The explanation of this fact has not yet been given, but it will certainly be of significance for both the physiological and the genetical problem.

There are some other data on the connexion between the resistance to different toxicants that are of importance for the two problems. BUSVINE (1953b) has shown that resistance to DDT can be quite independent of resistance to γ-HCH and chlordane. BARBESGAARD and KEIDING (1955) obtained some evidence indicating that the gene for DDT-resistance can contribute to the resistance to γ-HCH, although it is not indispensable for it. The question of the possible interrelations of resistance to different toxicants is further discussed in chapter VIII.

We can conclude that multiple genes are involved in the resistance to both DDT and γ-HCH. In the resistance to DDT sometimes genes can be distinguished that have a very large influence. Till now no investigations have been carried out dealing with the relations between the physiological and the genetical data. This will be an interesting field for further work.

B. Physiological data

There are two kinds of differences between resistant and susceptible strains. First those that are the cause of the difference in susceptibility, originated by the influence of the selection. These are treated in this

chapter. Second the differences that are not the cause of the resistance. There may be, for instance, differences of properties irrespective of the selection. They are an expression of the normal variations existing in these properties. This necessitates in general investigations with several susceptible and resistant strains in evaluating the significance of a certain difference for resistance (PRATT and BABERS, 1953). Differences that are not a cause of resistance may still be a result of selection. This will be the case if the genes for resistance are pleiotropic in their effect. They may have an influence on other properties parallel to that on resistance. Another possibility is that the property responsible for resistance, in its turn, causes all kinds of secondary differences. Probably the morphological differences described for the DDT-resistant flies are an example of pleiotropic effects (BIGELOW and LEROUX, 1954).

These possibilities make it very difficult to interprete differences found correctly. In general a difference can only be regarded as a cause of resistance if it lowers the amount of the toxicant that reaches the site of action or has an influence on the site of action itself.

A few remarks will be made here on this site of action and the mode of action of the insecticides under discussion. Only some general conclusions will be reported. WINTERINGHAM and BARNES (1955) may be consulted for a more detailed discussion.

It has been found that DDT affects the sense organs in insects (ROEDER and WEIANT, 1948). Besides, an action has been shown on the central nervous system (DRESDEN, 1949; FRITSCH, 1952; FRITSCH and KRUPP, 1952). It must be pointed out that in *Musca* the amount of DDT that reaches the head is of special importance for the lethal effect (FISHER, 1952; LEROUX and MORRISON, 1953, 1954; MORRISON and LEROUX, 1954). There are of course many sense organs in the head but an action on the brain seems most likely to be of primary importance in this insect. An exciting effect of γ-HCH on isolated central nerve cords has been shown in *Dytiscus marginalis* L. (FRITSCH, 1952; FRITSCH and KRUPP, 1952). An action on the sense organs has also been reported (LALONDE and BROWN, 1954) but is perhaps of less importance.

The primary effects that bring about this excitating influence of the two toxicants are not yet known. It is possible that the action depends rather upon their physical than upon their chemical properties. There is a theory according to which γ-HCH by its resemblance to inositol would disturb the action of this vitamin. However, this theory has not at all been proved. In the present state of our knowledge of the mode of action it is clear that only mechanisms have been studied that influence the amount of the insecticide at the site of action.

There are many kinds of differences that could possibly contribute to resistance. With respect to the resistance to DDT, differences in the

following properties will be discussed: behaviour, morphology, absorption, detoxication, excretion, other properties.

A. Behaviour

From a number of observations in the field there is evidence of a differential behaviour in susceptible and resistant strains (CHADWICK, 1955). By changes in resting habits treated surfaces might be avoided, while a decreased motility will decrease the chance of picking up a lethal dose. Experiments on these differences have not been carried out, however. An exception perhaps is the work of SMYTH and ROYS (1955) who found that a susceptible strain preferred a surface treated with DDT to an untreated one, whereas a resistant strain did not. This difference is perhaps a secondary effect, brought about by the resistance of the flies that depends on other differences. Thereby they may no longer undergo the stimulating influence of DDT and thus lose the power to discriminate between the two kinds of surfaces.

B. Morphology

A difference in morphology between the legs in a resistant and a susceptible strain was reported by WIESMANN (1947). He suggested that this would result in a smaller uptake of DDT. However, in other resistant strains such changes have not been found. From the fact that most resistant strains are also highly resistant upon application or injection of DDT, it may be inferred that differences in morphology are of minor importance only. It is not unlikely that the difference between the two strains of WIESMANN was but an accidental one.

C. Absorption

The process of uptake of outside DDT in the integument is indicated by the terms absorption or penetration. As the latter term can also be used for the process of passing through the integument we prefer here the word absorption. This process has been studied by many workers. In general DDT is applied to the thorax as a solution in ethanol or acetone. The solvent evaporates rapidly probably leaving the DDT partly dissolved in the cuticular waxes. After a certain period of time the insect is washed with a fat solvent. The part removed by a short washing is considered as outside, the remaining part as inside DDT. The absorption can easily be studied in this way. It will be apparent that there is the possibility that the absorption after contact will be different from that after application to the thorax. It might well be that there are differences in absorption by the legs which are not found when thoracic absorption is studied. The very large resistance that is often found after

application on the thorax, however, precludes the overlooking of an important difference.

STERNBURG, *et al.* (1950) applied dosages of 0.5 μg DDT on susceptible and resistant flies. During the first hours absorption was going on at about the same rate in both strains. In the resistant strain the dosage was taken up for the most part after 20 hours. In the susceptible strain the uptake decreased after 4 hours, a moment that coincides with the knock-down of the flies. After application of larger doses the absorption increases, but not proportionally with the dose. In this case appreciable amounts may be found on the outside even after two days.

Similar results were obtained by PERRY and HOSKINS (1951b). After application of 2 μg on a susceptible and two resistant strains 33, 42 and 45 % was absorbed respectively within 2 hours. When the susceptible flies were killed a decreased absorption was found.

WINTERINGHAM *et al.* (1951) applied 2 μg per fly of a radioactive Bromine analoge of DDT (2, 2-bis (*p*-bromophenyl) 1, 1, 1-trichloroethane). The difference in susceptibility between the two strains also applies to this compound. No difference in the rate of absorption was found.

PERRY, FAY, and BUCKNER (1953) studied absorption in 5 strains after application of 0.65 μg DDT. Although there are differences between the strains, they are not correlated with resistance. It may be concluded that susceptible and resistant strains have in general the same absorption. However, more recently PERRY has found some strains with an absorption rate of about ⅓ of that of susceptible flies (PERRY cited by KEARNS, 1955). In this respect it is interesting to note that BABERS and ROAN (1953) found a somewhat diminished rate of absorption of DDT in a resistant strain of *Blatella germanica* L.

It should be borne in mind that absorption may be quite different from penetration through the integument. Probably a large part of the DDT absorbed is retained by this organ, whereas only small amounts are carried by the haemolymph to other parts of the body.

STERNBURG and KEARNS (1950) for instance could not find DDT in other organs than the integument to which it was applied, provided that the mouth parts had been removed to prevent ingestion.

TAHORI and HOSKINS (1953) did find DDT in the internal organs. In their experiments however, oral ingestion was not precluded.

From experiments with C^{14}-labeled DDT, it is known that the amount of radioactive material that reaches the head is correlated with the result of the intoxication. (FISHER, 1952; LEROUX and MORRISON, 1953, 1954; MORRISON and LEROUX, 1954). If the circulation is impeded by ligatures around the cervix or pedicel the amount of DDT or metabolites that reaches the head decreased, and results in a lower mor-

tality. As the only known metabolite is the non-toxic DDE, it seems probable that for the intoxication the amount of DDT penetrating through the integument is important. In view of the results of STERNBURG and KEARNS cited above, this may be only a fraction of the dose absorbed. Whether there are differences in the penetration through the integument in susceptible and resistant strains is not known.

The amounts of DDT that are absorbed after application are not at all proportional to the amounts applied (BUSVINE, 1951). Especially in higher dosage ranges the amount absorbed increases only very little with increasing doses. If the ratio between the LD's 50 of susceptible and resistant strains as found with the application method is taken as a measure of resistance, very high values may thus be found. Much smaller values are obtained, however, when application is carried out in such a manner that the absorbed amount is more proportional with the dose applied. BUSVINE found a ratio of 16 between the LD's 50 if DDT was applied in mineral oil, of 300 after application in acetone.

D. Detoxication

When a colorimetric method for the estimation of small quantities of DDT had become available metabolism was studied in many insects. In most cases the non-toxic dehydrohalogenation product 2,2-bis(*p*-chlorophenyl)1,1-dichloroethylene (DDE) appeared to be found. This metabolite can be estimated in the same colorimetric method simultaneously with DDT.

STERNBURG and KEARNS (1952) demonstrated the formation of DDE in some natural resistant insects, *Melanoplus differentialis* Thomas and *femur-rubrum* De Geer, *Epilachna varivestis* Mulsant and *Argyrotaenia velutinana* Walker, VINSON and KEARNS (1952) found a conversion to DDE in *Periplaneta americana* L, BABERS and ROAN (1953) in a susceptible and resistant strain of *Blatella germanica* L. Many authors have studied the formation of DDE in DDT-resistant houseflies (STERNBURG *et al.*, 1950; STERNBURG and KEARNS, 1950; PERRY and HOSKINS, 1951b; FLETCHER, 1952; BABERS and PRATT, 1953; PERRY, FAY, and BUCKNER, 1953; TAHORI and HOSKINS, 1953). Sometimes it was also found in susceptible strains (PERRY and HOSKINS, 1951; BABERS and PRATT, 1953; PERRY, FAY, and BUCKNER, 1953).

BABERS and PRATT (1953) injected DDT solutions in acetone in susceptible and resistant flies. In the resistant flies increasing doses, up to 60 μg, were injected, but no increase in the mortality resulted, whereas the formation of DDE diminished. They concluded from these results that the formation of DDE is blocked by high doses of DDT and that consequently detoxication of DDT cannot be an important factor in resistance. Their results, however, can probably be better explained in

another way. After the injection in acetone especially in the higher concentrations (up to 28 % was injected) the DDT may not be distributed through the body but probably crystallizes at the site of injection. The smaller conversion to DDE could thus be explained by a smaller supply of DDT to the detoxicating tissues.

In most investigations into the influence of detoxication on resistance the DDT was applied topically. Of the amount absorbed by the two strains more DDE is formed by the resistant strain. PERRY, FAY, and BUCKNER (1953) found a very good correlation between the formation of DDE and the degree of resistance. They therefore proposed to use an index as a measure of the degree of resistance to DDT. It was defined as the percentage of the absorbed DDT dehydrochlorinated to DDE 2 hours after the application of 5 to 10 μg.

The results of these experiments indicate that detoxication of DDT is very likely to be an important factor. However, the experiments remain open to the criticism that DDE formation may be suppressed in the susceptible strains by the toxic action of DDT. The difference in the formation of DDE could therefore be a result rather than a cause of resistance (CHADWICK, 1952; BABERS and PRATT, 1953).

BABERS and PRATT tried to evade this difficulty by studying the DDE formation after injection of small dosages of DDT (0.3 μg/fly). They found more DDE in the resistant than in the surviving susceptible flies. However, the authors were unable to study the strains in the same condition of poisoning, although they pointed out that this would have been necessary.

The larger formation of DDE being a cause and not a result of resistance, was proved when STERNBURG *et al.* (1953) demonstrated DDE formation to be present in homogenates of resistant flies but not of susceptible flies. In a more recent work (STERNBURG *et al.*, 1954) a method was described for the partial purification of the enzyme, (DDT dehydrohalogenase) and results with more strains were reported. A fairly good correlation has been found between the degree of resistance of the strains and the enzymatic activity of the homogenates.

Although it has been proved by these investigations that the formation of DDT is an important factor in resistance it does not give information on the possible existence of other mechanisms. When with some compounds it proved possible to inhibit the detoxication to DDE, more data on this question could be obtained. A number of non-toxic compounds was found that enhance appreciably the action of DDT to resistant flies, if administered simultaneously. The physiological action of two of these synergists has been investigated *viz.* of piperonylcyclonene (PERRY and HOSKINS, 1950, 1951 a, b; FULLMER and HOSKINS, 1951; WINTERINGHAM *et al.*, 1951) and of 1,1-bis(*p*-chlorophenyl)methylcar-

binol (DMC) (Perry, Mattson, and Buckner, 1953; Moorefield and Kearns, 1955). These synergists inhibit the detoxication to DDE and thereby increase the toxic effect of DDT in resistant flies.

By applying piperonylcyclonene the LD 50 of a DDT-resistant strain could be lowered from 0.5 μg per fly to 0.078 μg, while the LD 50 of the susceptible strain used was 0.054 μg (Perry and Hoskins, 1951a). The LD 50 of another multi-resistant strain could be reduced by DMC from 17 μg to 0.4 μg. In these experiments the LD 50 of the susceptible strain was 0.3 μg (Perry, Mattson, and Buckner, 1953). Increasing amounts of DMC gave a parallel increase in inhibition of DDE formation and in susceptibility of the resistant flies. Moorefield and Kearns (1955) confirmed the inhibiting action of DMC on the DDT-dehydrohalogenase in *vitro*-experiments.

These results proved that in the strains studied the larger part of the resistance is brought about by the detoxication of the DDT. As will appear below there are indications that at least in some other strains a second important mechanism is present. Research in more strains with the aid of inhibitors is therefore very desirable.

There is only little information on the important question whether the DDT dehydrohalogenase is present in all tissues or only in certain organs. From the work of Sternburg and Kearns (1950) it is known that DDE formation can take place in the integument and in the digestive tract. Whether it is also present in other organs is not known.

E. Excretion

Excretion of DDT taken up by contact seems not to occur. Some investigators (Sternburg *et al.*, 1950; Perry *et al.*, 1955) found DDT and DDE in beakers, in which resistant flies had been kept for some days. Whether this was present in the excreta or may have been derived from the integument has not been investigated. LeRoux and Morrison (1954) found 24 hours after the application of C^{14}-labeled DDT 2% of the activity in the excreta of a resistant, 0.9% in those of a susceptible strain. By means of this technique Terriere and Schonbrod (1955) found no excretion of DDT. An excretion of metabolites was shown, but in this respect no differences between a susceptible and a resistant strain could be demonstrated.

F. Other properties

Although it has been proved that the detoxication of DDT is an important factor in resistance, there is strong evidence for the existence of one or more other mechanisms. Sacktor (1949, cited after Chadwick, 1952) and Johnston (1951) found an inhibiting effect of DDT on cytochrome oxidase. In a resistant strain Sacktor (1950) measured

a cytochrome oxidase activity 50 % higher than in a susceptible strain. A two times higher cyanide-insensitive respiration was present in the pupae (SACTOR, 1951). He suggests that these differences will enable the resistant strain to maintain its respiration in the presence of DDT. However, it is not probable that the toxic action of DDT depends on the inhibition of the respiration. JOHNSTON (1951) for instance, found a similar effect for the non-toxic DDE and SACKTOR for the *o–o'* and *o–p'* isomers of DDT (cited after CHADWICK, 1952). ANDERSON *et al.* (1954) studied the inhibitory action of DDT and related substances on the succinic-acid oxidase system of susceptible and resistant flies. They concluded that this inhibition is no primary factor in the toxicity of DDT. Recently studies have been made of the cytochrome oxidase activity of 10 strains of different degrees of resistance (PERRY and SACKTOR, 1955). No correlation was found with resistance. It has not been proved therefore, that the differences found are of importance for resistance.

WIESMANN (1954, 1955) investigated the tremors appearing in isolated legs of susceptible and DDT-resistant flies upon treatment with the vapour of benzene. In both strains tremors occurred but in the susceptible strain they soon ceased as the legs were too heavily damaged. After a treatment with diphenyl and DDT, more and stronger tremors occurred in the legs of the susceptible than in those of the resistant flies. From these experiments WIESMANN concluded that there is probably a difference in the permeability of the nerve tissues. The small benzene molecule would still be able to penetrate the nerves of the resistant flies, whereas the larger diphenyl and DDT molecules could only do so to a slight degree. As he also found differences in the uptake of vital dyes by hypodermal cells, WIESMANN thinks it probable that there are differences in the permeability of all tissues. Extension of these investigations on more strains in order to ascertain whether there is a correlation with resistance, is very desirable. Whether the relatively specific DDT resistance can be explained by a non specific change in permeability is still to be studied.

It has often been noticed that in the resistant insects, in spite of its degradation to DDE, more DDT can be present than is necessary to kill the susceptible flies (WINTERINGHAM *et al.*, 1951, FLETCHER, 1952). This, however, does not imply that there are other defence mechanisms, as of these strains nothing is known about the concentration of DDT at the site of action. The insecticide may be present in tissues where it is relatively harmless, *e.g.* in the integument to which it has been applied or in the fat body, whereas the detoxicating mechanism may prevent the accumulation of a lethal concentration at the site of action.

It has been demonstrated by some workers that there is a difference

between the nervous systems of susceptible and resistant flies. PRATT and BABERS (1953a) applied DDT in an emulsion directly to the exposed ganglia of flies of both strains. Flies of the resistant strain showed less tremors in their legs than those of the susceptible strain. WEIANT (cited after CHADWICK, 1955) found upon injection of DDT hyperactivity in the sensory nerve fibers of the legs of susceptible, but not of resistant flies. WIESMANN (1955) demonstrated a rapid diminishing of the proboscis-extension-reflex in susceptible flies treated with DDT, in resistant flies the reaction was hardly influenced. These facts may indicate the presence of a mechanism other than detoxication. A detoxication in the nervous system or in surrounding tissues might, however, also be the mechanism involved. Perhaps experiments in which the detoxication is inhibited may decide the question whether detoxication or another mechanism is involved.

We have seen already that resistance to DDT can be overcome largely by the use of synergists. However, after a few generations of selection the DDT-resistant strains are able to develop resistance to the mixture of DDT and the synergist (MARCH *et al.*, 1952). This finding could possibly be explained by assuming another mechanism. MOOREFIELD and KEARNS (1955) showed that these strains have a still higher DDT-dehydrohalogenase activity than the strains resistant to DDT alone. Therefore, the assumption of another mechanism again is not imperative in this case.

BUSVINE (1951, 1953 a,b) has pointed out that there are probably at least two mechanisms of importance for DDT resistance. He studied two strains, one resistant both to knock-down and to kill (Strain I), the other susceptible for knock-down and resistant to kill (strain S). Both strains can degrade DDT at about the same rate. Strain I was also resistant to some compounds that cannot be dehydrohalogenated *viz.* 1,1-bis (*p*-methoxyphenyl)1,1,1-trimethylethane and 1,1-bis (*p*-chlorophenyl), 2-nitropropane, strain S was not resistant to these sub-substances (HARRISON, 1953). It is probable, therefore, that resistance in strain S depends on its detoxication mechanism, but that in strain I in addition another mechanism is present. The fact that in strain I a special gene is involved in the resistance to knock-down (p. 5) is also an argument in favour of such a mechanism.

It must be pointed out that this mechanism does not bring about resistance to γ-HCH, chlordane and related compounds. Therefore it must have a great specificity, just as in the case with the detoxication. The resistance to different toxic DDT-analogues in strain S shows a correlation with the rate of their dehydrohalogenation by alkali *in vitro*. This is not found in strain I. In experiments with these substances KEARNS (1955) did not find a correlation between the dehydrohalogena-

tion *in vitro* by the enzyme and by alkali. The explanation of the correlation found by BUSVINE can therefore not yet be given.

KEARNS showed that methoxychlor is degraded by DDT-dehydrohalogenase, but at a lower rate than DDT. If the breakdown were the only existing mechanism, the same ratio would be expected between resistance to DDT and to methoxychlor in different strains. However, a strain with a higher as well as one with a lower resistance to methoxychlor than to DDT have been selected (BRUCE and DECKER, 1950; DECKER and BRUCE, 1952). So, either there must be enzymes with different specificities to these compounds in the two strains, or one or more other mechanisms are also involved here.

SUMMARY AND PROBLEM

DDT resistance is brought about by at least two different important mechanisms. One of these is detoxication to DDE, the nature of the other one is not yet known. There are indications that this mechanism is of special importance in resistance to knock-down, which might be caused by a changed permeability of the nervous tissues. Besides these two mechanisms there are probably some other properties of less importance involved.

From the study of the literature it is clear that resistance to DDT has been very intensively investigated. This research is being continued in many laboratories. Resistance to γ-HCH, however, has been given little attention so far. The same problem as with DDT presents itself here: What are the causes of the differences between susceptible and resistant flies? On page 7 we have seen already that the scanty knowledge of the mode of action limits the research to the factors that influence the amount of the toxicant at the site of action. The factors can be divided in two groups: those with an influence on the amount of the toxicant in the insects and those that influence the distribution in the insect. With the available estimation method of HCH, it proved possible to study the first group of factors. Therefore the following two questions will be studied:

1. Are there differences in absorption, detoxication and excretion of importance to the resistance?
2. What is the relative significance of the differences found?

III. MATERIAL AND METHODS

BREEDING OF MUSCA DOMESTICA

For the breeding of houseflies a choice can be made between a fairly large number of methods. The method described here distinguishes itself from many others by the

fact that it is practically odourless, so that it can be used in small closed rooms and incubators. It is very suitable for obtaining a continuous output of not too large numbers of flies of many strains.

The culture was kept at 25° C and 70 % relative humidity. The flies used to obtain eggs were kept in breeding cages of 40 × 22 × 23 cm. One side is provided with a sleeve, through which flies and food can be introduced into the cage. In every cage some hundreds of flies can be kept. They are supplied with water and food consisting of a mixture of milkpowder and sucrose with some yeastpowder. To obtain eggs a disk is introduced with a mixture of the following composition: 250 g skimmed milk-powder, 16 g malt extract, 12 g yeast powder, 1000 g water, 200 g papertissue. This mixture must be brought to a pH of about 8.

The flies deposit their eggs in crevices in the material. To obtain the same conditions in the rearing jars it was necessary to administer approximately equal numbers of eggs to every jar. To this end the eggs were suspended in water and brought into small tubes in which a volume of the desired number was allowed to settle down. The rest was discarded. The measured amounts of eggs were put into the jars.

The breeding medium consisted of dog-biscuit, inoculated with a yeast suspension and mixed with a small amount of peat-dust to obtain a loose mass. To prevent the growth of moulds 2 ml of a 10 % solution of *p*-hydroxymethylbenzoate in alcohol was added to every liter of medium. About 2 l of the mixture was used in each jar. The eggs were introduced and covered with a layer of wettish peat-dust, in which the grown larvae will pupate. Per jar about 500 flies can be bred. During the larval period the temperature in the jars rises some degrees above that of the room. About 11 days after the introduction of the eggs the flies emerge. They were used in the experiments without previous feeding, from 0 to 2 days after emergence.

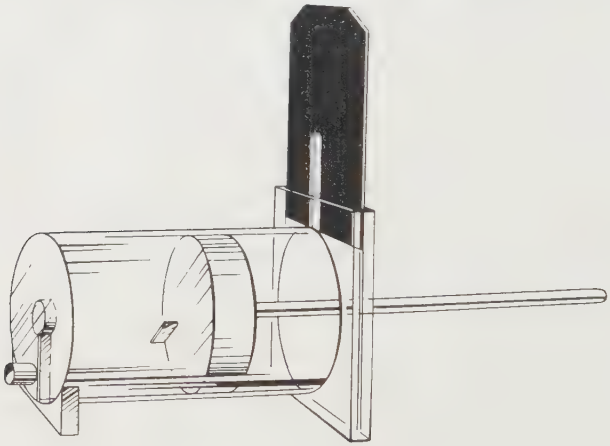

Fig. 1. Apparatus for handling flies.

The flies were transferred from the jars into test tubes by means of an apparatus of perspex (Fig. 1) of which a somewhat different type has already been described by VAN TIEL (1955). It consists of a cylinder closed on one side by a slide. It can be fitted on a jar; by opening the slide and removing the cloth on the jar the flies are admitted to the cylinder. By pushing in a plunger the flies are assembled in the opposite side of the cylinder from where they can enter the test tube through an opening. On its interior side the plunger is fitted with a radiar placed strip of metal, 1 cm high. The front

of the cylinder is also provided, on the inside, with such a strip. The plunger is pushed forward in such a position that the strips come to lie against each other. By turning the plunger the flies are driven together in a narrowing sector, so that they are forced to enter the test tube through the opening.

ADMINISTRATION OF THE INSECTICIDE

Both for the estimation of the susceptibility and for the study of the fate of γ-HCH in the insect, the toxicant can be administered in three ways: by means of contact, application or injection. These methods each have their specific advantages and disadvantages which determine which should be used in every case.

A. Contact

The contact method was used in the cases in which large numbers of flies had to be treated, for instance for the selections and the estimation of the susceptibility. In most cases the presented surface consisted of a peace of filter paper of 6 × 15 cm, to which the γ-HCH had been applied as a solution in acetone. In the biological estimations where very small quantities had to give a high mortality, the toxicant was presented as a residue in a very thin layer of paraffin oil.

B. Application

An acetone-solution of γ-HCH was applied topically onto the dorsal thorax by means of a micro-loop method. In this method a loop of wire is dipped into the solution of HCH and then brought into contact with the dorsal thorax of a fly. The solution rapidly spreads on a large part of its surface. If care is taken that the position of the loop and the speed of dipping are as constant as possible the method is accurate enough for employing it for the estimation of the susceptibility and the study of absorption and metabolism of the toxicant. Throughout the work the same loop was used which delivered approximately 0.5 μl. As the volume applied depends upon the manner in which the loop is handled, in every experiment a separate group of flies was treated to estimate the exact amount of HCH that had been administered. During the application the flies were narcotized by CO_2.

C. Injection

For the injections oily emulsions were used, as these have certain advantages over solutions in acetone or oil. Disadvantages of the use of acetone are its toxicity and the possibility that the toxicant cristallizes at the site of injection. Disadvantages of the use of oil are its high viscosity and the fact that the insecticide probably leaves it only slowly through diffusion or as the oil is degraded in the tissues.

Advantages of emulsions are their rapid distribution with the haemolymph and the very large surface of the oil, by which the diffusion of the toxicant will be relatively rapid. Sometimes they have the drawback that the amount of insecticide that can be dissolved in the oil is too small and that difficulties may arise in the reproducibility of the degree of dispersion.

In most cases emulsions were obtained according to DRESDEN (1949) by mixing 1 g of gum arabic with 1 ml of peanut oil with a small amount of physiological salt solution. This is ground in a mortar until a filmy mass is obtained, after which the mixture can be diluted further with the saline to the desired volume. The composition of the saline was as follows (modified after DRESDEN, 1949): 9.32 g NaCl + 0.77 g KCl + 0.50 g $CaCl_2$ + 0.180 g $NaHCO_3$ + 0.010 g NaH_2PO_4 to 1 l H_2O.

In this way it is not possible to increase the ratio between the amounts of oil and gum. In cases where high oil concentrations had to be used in order to dissolve enough

HCH so much gum would have been needed that the resulting emulsion would become too viscous. In these cases another method was therefore adopted. The gum was solved in the salt solution, the peanut oil added and the mixture was emulgated in a Waring blendor. These emulsions are always less stable than those obtained in a mortar. To prepare emulsions no use should be made of substances that lower the surface tension as this would cause the injected emulsion to spread on the fatty insect cuticle. This difficulty never occurred in the emulsions with gum arabic.

The injections were carried out with constriction pipettes described by LINDERSTRØM-LANG and HOLTER (1940). The pipet is first filled to somewhat above the constriction. Then, by gently blowing, the surface is brought back into the constriction. In this way these pipets could very accurately deliver a small volume (0.3 to 1 μl).

The tip of the pipet is introduced into the thorax a little before the wing base. By blowing a little harder it can be emptied between the flight muscles. The injections were carried out under a dissection microscope while the flies were anaesthetized by CO_2.

WASHING TECHNIQUE FOR SEPARATING OUTSIDE AND INSIDE HCH

To study the absorption after application of HCH onto the thorax it is neccessary to be able to estimate outside and inside HCH separately. As the distinction obviously is a little arbitrary the question arises as to what can be best considered to be outside and inside. In practice there appears to be a simple solution of this question. Just as with DDT the HCH present can be devided sharply into a part that can be dissolved by a washing with a fat solvent *e.g.* methylenechloride, and a part that cannot. Probably all HCH present in the waxy layer is washed off, and perhaps also a part present in deeper layers. This part will be called outside HCH. The part which cannot be washed off will be called inside HCH. This distinction is useful for our purpose, if it is kept in mind that "inside" HCH may yet be present in the integument to a large extent, and that the amount that reaches the internal organs is not known.

To find a suitable washing technique HCH was applied to groups of 70 flies. After a certain period of time they were narcotized and placed on a perforated glassplate in a funnel. They were rinsed with successive portions of 10 ml of methylenechloride. The HCH present in the separate washings and in the rinsed flies was estimated separately with the method of SCHECHTER and HORNSTEIN (p 20). In table 1 it is shown that the amount of γ-HCH rapidly declined in the successive portions, practically nothing being removed after the third washing. It was decided, therefore, to wash with three portions of 10 ml of methylenechloride, and to combine them for the estimation of outside HCH. In the rinsed flies the inside HCH could be estimated.

The data in table 1 on the other isomers are scarce. However, they are strengthened by the fact that shortly after application, practically all of the HCH is found in the wash. This shows that the washing is adequate to remove outside HCH. From table 1 it can be seen that for these isomers there also remains a certain amount in the flies that cannot be washed off. For that reason the same washing technique was chosen for the α- and δ-isomers.

It could be suspected that with this method outside HCH might be carried to the inside of the flies by the washing fluid. This is not probable, however, for the following reasons:

1. The methylenechloride has only a very short-lived contact with the flies.
2. In flies washed shortly after application only very little HCH was found.
3. A group of flies was washed with a portion of methylenechloride containing 100 μg of γ-HCH, and the remaining outside HCH was removed with pure methylenechloride. No inside HCH was found.

Whether, reversely, some HCH might be extracted from the inside organs of the flies, was studied by washing HCH-injected flies. Half an hour after injection of γ-HCH 18 μg was present in the flies, nothing in the wash, which shows that extraction at least plays no important part. These experiments prove that the technique is suitable for the study of the absorption.

TABLE I

HCH obtained by successive washings with 10 ml of methylenechloride

	70 flies of strain R_2 applied with 0.75 μg per fly. Washed after 75 minutes		70 flies of strain R_2 applied with 0.5 μg per fly. Washed after 3 hours	70 flies of strain S applied with 1 μg per fly. Washed after 2 hours
Wash no.	*μg of γ-HCH*		*μg of α-HCH*	*μg of δ-HCH*
1	16	15	5	13
2	6	11	2	2
3	1	7	1	1
4	0	2	–	1
5	–	2	–	–
10	0	–	–	–
Inside	18	16	12	36

ESTIMATION OF HCH

In order to investigate the absorption and detoxication of HCH it is necessary to dispose of an estimation method with a high specificity and sensitivity. Especially where a large number of estimations must be done, it is desirable that the method is as simple as possible. The specificity of the method is very important in the study of the metabolism of the toxicant, as only with a highly specific method it becomes unlikely that breakdown products are estimated as HCH. A high sensitivity enables experiments with relatively small numbers of animals, so that individual application and injection become possible.

Of the chemical estimation methods of HCH some colorimetric methods are suitable for physiological work (Armstrong *et al.*, 1951; Phillips, 1952; Schechter and Hornstein, 1952). Moreover, it is possible to estimate γ-HCH by means of bioassay methods (Sherman and Norton, 1948; Hoskins and Messenger, 1950; Hoskins *et al.*, 1952). The colorimetric methods are fairly specific. Only the method of Armstrong *et al.* has up to now been used for physiological work. The other methods have been used for the estimation in food contaminated with γ-HCH.

The sensitivity of the methods of Phillips, and of Schechter and Hornstein is somewhat higher than that of the method of Armstrong *et al.*, the Schechter-Hornstein method is the simplest of the three. This simplicity is due to the fact that in many cases the material in

which the HCH must be estimated can be used without any preliminary extraction, whereas in the other methods the HCH has to be extracted from the organic material. The SCHECHTER-HORNSTEIN method was therefore chosen for our work. In a single case use was made of the bioassay method of HOSKINS *et al.* (1952). This method has a very high specificity and sensitivity. As it is rather laborious its application was limited to an examination of the specificity of the SCHECHTER-HORNSTEIN method (p. 22).

CHEMICAL ESTIMATION METHOD OF SCHECHTER AND HORNSTEIN

A. *Principle of method*

The HCH is dechlorinated by Zn in boiling acetic acid. The benzene formed is nitrated to *m*-dinitrobenzene, which, in the presence of strong alkali, gives a violet coloured reaction product with methylethylketone. The intensity of this violet colour is estimated.

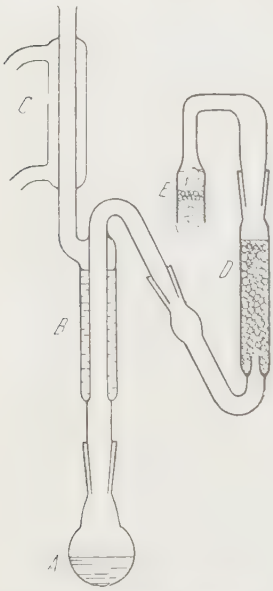

Fig. 2. Apparatus used in the determination of HCH. A-reaction flask; B-cooler with trichloroethylene; C-water cooler; D-nitrating tube; E-tube with soda lime.

B. *Technical details*

The method was carried out largely as described by SCHECHTER and HORNSTEIN. A difference was the use of the apparatus described by REITH for the qualitative estimation of HCH in vegetables (Fig. 2). This consists of a flask (A) of 15 ml in which the material, *e.g.* 1 g of flies was brought into 5 ml of acetic acid. After addition of ½ g of Zn dust (HORNSTEIN, 1952) and 1 g of malonic acid the flask was connected to a small cooler (B) kept at 90° C. This was achieved by a stream of water from a thermostat or by filling the cooler with trichloroethylene which boils at that temperature and flows back on the water cooler (C). The mixture in the flask is boiled for 45 minutes. In the cooler B the acetic acid is refluxed whereas the benzene is carried by the gas stream (CO_2 from the malonic acid, H_2 from the Zn with acetic acid) to the nitrating tube (D).

The nitrating acid was treated as described by SCHECHTER and HORNSTEIN. The acid is diluted and the *m*-dinitrobenzene is extracted with ether. This is neutralized with NaOH and the

water is removed with a saturated NaCl solution. After evaporation of the ether the colour reaction is carried out.

In contrast with the SCHECHTER-HORNSTEIN method the colour was not estimated after 20 min. at 5650 Å, but after 50 min. at 5800 Å. Although the sensitivity is somewhat lowered by this change, a greater accuracy was obtained as sometimes an interfering orange colour was formed, which has less influence under these circumstances. The colour was estimated by means of a Spekker photoelectric absorptiometer.

C. *Addition of the material*

As a rule HCH was estimated in ½ to 1 g of flies or in small amounts of solvents *e.g.* methylenechloride or carbontetrachloride. The flies were directly added to the acetic acid, and in the flask crushed with sand by a glass rod. This method proved more accurate when a preceding extraction was omitted. The solvents in which HCH had to be estimated (mostly about 30 ml) were evaporated after adding a drop of paraffin in the flask, upon which the acetic acid was added.

D. *Estimation of different isomers*

With the method described α- and γ-HCH can be estimated. For the estimation of β- and δ-HCH, which are less readily converted to benzene, good results were obtained by boiling the reaction mixture for 90 minutes with 1 g of Zn dust. The use of very fine Zn dust is of special importance in this case.

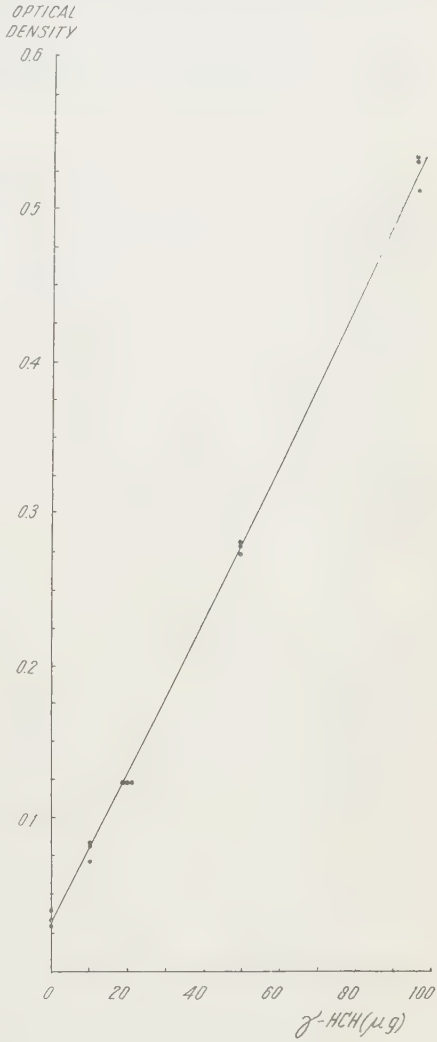

Fig. 3. Standard curve.

E. *Calibration of the method*

Before the method could be employed for unknown quantities of HCH,

the relation between known amounts of HCH and the colour produced had to be established. In every estimation 1 g of flies was added, to make the circumstances identical with those in the estimations of unknown amounts. From Fig. 3 it can be seen that the relation between amounts up to 100 μg of γ-HCH is linear, the extinction per μg being 0.049. At different times the value of this conversion factor was checked. For the α-, β- and δ- isomers the same extinction was found, provided that the precautions were taken as mentioned for the β- and δ- isomers.

F. *Accuracy of the method*

The accuracy depends to a large extent on the reproducibility of estimations without HCH. Taken over longer periods of time these blanks showed considerable variations. They were largely due to impurities of the chemicals used. The fly-material itself remarkably had no appreciable blank-value; blank estimations with and without flies gave the same results. Within short periods of time *e.g.* a few days, the blanks did not vary more than corresponds with about 3 μg of HCH if the same stocks of chemicals were used. In the experiments with low dosages the accuracy is practically wholly determined by these variations. With higher doses the variation in the colour produced per μg also plays a part. In general the variations between the estimations do not exceed 10%, but mostly they are smaller.

G. *Specificity of the method*

As is described in chapter V there is evidence that metabolism of HCH in the flies does occur. It is possible to calculate the rate of metabolism from a decrease in the colour intensity produced in the SCHECHTER-HORNSTEIN method, provided that the method is specific for HCH. Otherwise there might be the possibility that degradation products still produce the colour, so that the breakdown appears smaller than it really is. Therefore some experiments were carried out to study the specificity of the method.

1. Reaction of related compounds

From the fact that animal material *e.g.* the flies give a very low blank it is already apparent that the reaction is fairly specific. Moreover, the following substances were found to give no colour in the reaction: phenol, trichlorobenzene, hexamethoxycyclohexane, mesoinositol and monochlorocyclohexane (VAN ASPEREN and OPPENOORTH, 1954).

2. Bioassay of γ-HCH

The bioassay method is highly specific and can therefore be used as a check on the specificity of the SCHECHTER-HORNSTEIN method. To this

end γ-HCH is administered to the flies and after some time the amount in the flies is estimated both by bioassay and the SCHECHTER-HORNSTEIN method.

The γ-HCH is first extracted from the flies, the extract is purified, the purified extract is administered to specimens of *Drosophila* in a contact method.

In a mortar 70 flies to be used in both estimation methods were ground up with sand and sodiumsulfate. The powder was brought on a filter and extracted by pouring 25 and 2 × 12.5 ml of methylenechloride over it. The methylenechloride was evaporated.

In order to purify the extract it was solved in 5 ml of carbontetrachloride. This was gently pressed through a column of 3 g of silicagel (with 2 % water) mixed with 2 ml of oleum (modified after BRADBURY and STANDEN, 1954). The fat is removed by this column, whereas the γ-HCH quantitatively appears in the eluate.

From the eluate dilutions were made and tried in the contact method of HOSKINS *et al.* (1952). The insects used, however, were specimens of *Drosophila* instead of houseflies. In this method a small amount of liquid paraffin is added to the solvent with the γ-HCH. The fluid is pipetted into the jars, that are rotated, leaving only the γ-HCH in a thin layer of paraffin on the walls, 2 μl on a surface of 80 cm^2. The jars were provided with a filter paper to take up any moisture, and a peace of cotton wool with a solution of sugar as food. In every jar 20 specimens of *Drosophila* were brought, 3–5 days of age. Deads were counted after 24 hours at 25° C.

Results. To 70 flies, of strain S and R_2 each, 1 μg of γ-HCH was applied per fly. The flies were kept for 3 hours at 25° C, after which the outside HCH was removed. Half the number of flies were used in the chemical method, the other half for bioassay. In the bioassay two series of jars were made: one series was obtained by adding known amounts of γ-HCH to the eluate of untreated flies ("standard series"). The other was made by dilution of the eluate of the treated series ("unknown series"). For this purpose it was assumed that the "γ-HCH" found with the chemical method was indeed γ-HCH. The dilutions were made so as to obtain the same concentrations as in the standard series. If it should be found, that the dose-response curves of the two series were different, the assumption would have been wrong. If they were the same, a strong argument for the specificity would have been obtained.

Fig. 4 shows that the dose-mortality curves are indeed very much the same. By bioassay and with the SCHECHTER-HORNSTEIN method the results of table 2 were obtained.

These values agree sufficiently well to justify the conclusion that the chemical estimated "γ-HCH" is indeed toxic and therefore is γ-HCH.

There is still the possibility that breakdown-products are formed that cannot be chemically estimated but are yet toxic. These substances should be estimated, in principle, by the bioassay method. This is not

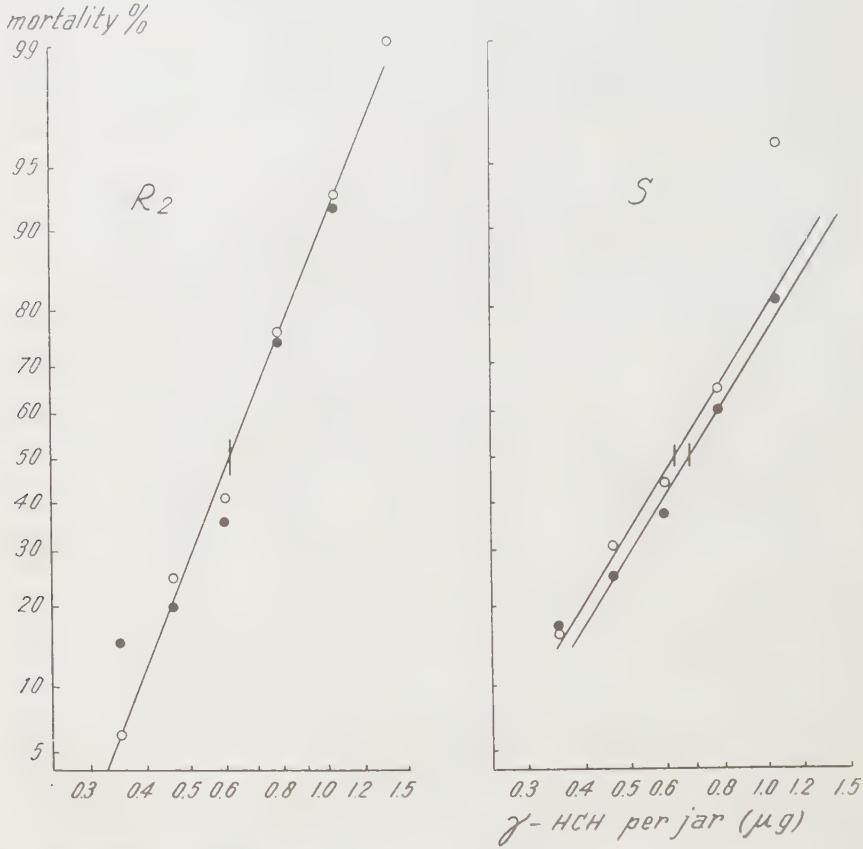

Fig. 4. Bioassay of amount of γ-HCH present in flies of strains S and R_2, 3 hours after application. Dots: Standard series. Circles: Extracted γ-HCH. Dilutions made on assumption that the chemically estimated value is correct.

TABLE 2

Results of chemical determination and bioassay of γ-HCH present in 70 flies, 3 hours after application of 1 μg per fly.

Strain	*Schechter-Hornstein method*	*Bioassay*
S	43	40
R_2	22	22

probable, however, as this method has a high degree of specificity by the purification of the extract with the oleum.

STRAINS

Eight strains were used, varying in susceptibility to γ-HCH from very susceptible to very resistant. Their resistance, origin and previous history, both in the field and in the laboratory are mentioned in table 3. In chapter IV their degree of resistance will be discussed in more detail. The measure of resistance mentioned in the table is expressed as the time in hours needed to give 50 % knock-down, when the flies are kept on a piece of filter paper with 1 mg of γ-HCH.

The strains U_2, R_1, and R_2 were in each generation selected with γ-HCH. For this purpose 80 flies where kept overnight in a jar containing a piece of filter paper with 250 μg of γ-HCH. The resulting mortality was about 30 %.

TABLE 3

Resistance to γ-HCH, origin and selections of the strains.

Strain	*ET 50 in hours*	*Origin*	*Obtained from*	*Selections*
S	0.16	?	Dr. N. v. Tiel. Koninklijke/Shell Laboratory, Amsterdam	None
MK	0.19	Netherlands	Prof. D. J. Kuenen, Zoological Laboratory, Leiden	DDT in the field
Bl	0.25			None
U	0.36	Uruguay	Dr. J. R. Busvine, London School of Hygiene and Tropical Medicine	DDT and γ-HCH in the field
E	1.03	England		
U_2	2.12	—	Selected from U	DDT and γ-HCH in the field. Selection in the lab. with γ-HCH
R_1	4.12	Denmark	Dr. J. Keiding. Government Pest Infestation Laboratory, Springforbi, Denmark	DDT, γ-HCH, Chlordane and toxaphene in the field. Selection in the lab. for DDT susceptibility and γ-HCH resistance.
R_2	5.16			DDT, γ-HCH, chlordane and toxaphene in the field. Selection in the lab. with DDT, γ-HCH and chlordane.

IV. ESTIMATION OF SUSCEPTIBILITY

The susceptibility of the strains can best be estimated with a method in which the administration of the toxicant is the same as in the physiological investigations. For instance, if the relation is studied between the breakdown of injected doses and the degree of resistance, the latter cannot be measured with an application method, as differences in absorption might play a part. Likewise, in studying the relation between physiological properties after application and resistance, misleading results might be obtained in measuring resistance with a contact method, for in this case differences in behaviour might have an influence. Only if the results of different methods can be shown to be the same, it is justified to apply a more suitable method for the estimation of resistance instead of the method used in the physiological work.

A. Injection

The susceptibility of the strains S, R_1, and R_2 was estimated after injection. The injected flies were kept at 25° C for 24 hours, after which deads were counted. In table 4 the results are shown. The LD's 50 of the strains S, R_1 and R_2 were 0.025; 0.58 and 0.85 μg per fly, respectively.

TABLE 4

Percentage of mortality of 10 flies, 24 hours after injection of 0.33 μl of an emulsion of γ-HCH in peanut oil.

Dose, μg / fly	*Strain*		
	S	*R_1*	*R_2*
0.016	20 40		0
0.062	80 90		0
0.27			0
0.34		30	
0.54		40	30
0.82		70 80	40 50 60
1.19			70
1.36			100

B. Application

Of the 8 strains used the susceptibility for γ-HCH was estimated by determining the time elapsing before knock-down occurred after application of 25 μg to the thorax in 20 flies of each strain. In fig. 5 the results are plotted as the logarithm of time against the percentage that was knocked-down.

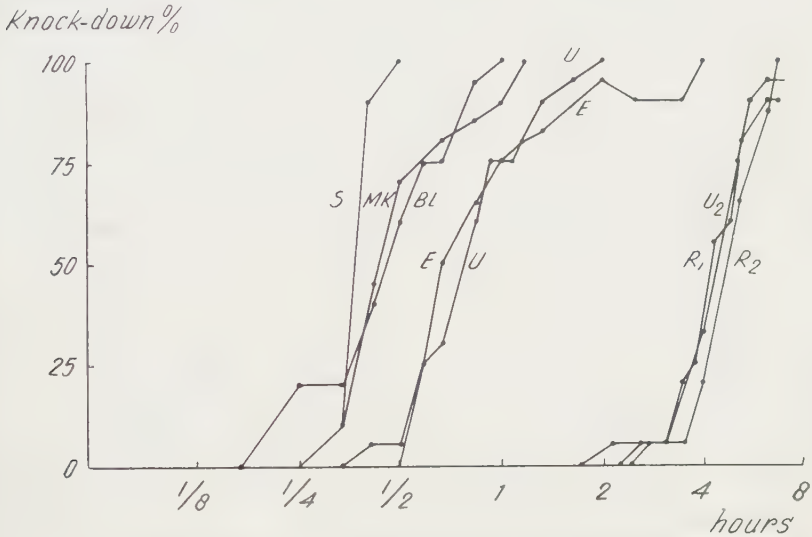

Fig. 5. Knock-down upon application of 25 μg of γ-HCH onto the thorax.

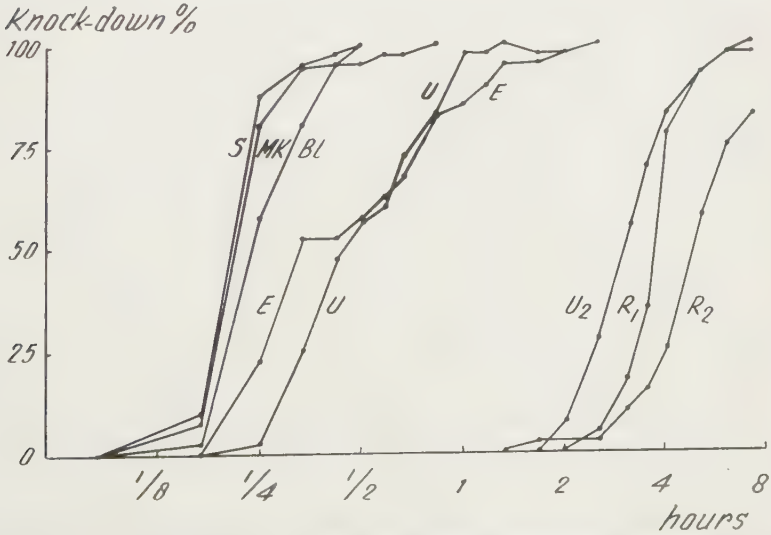

Fig. 6. Knock-down upon contact with a filter paper impregnated with 1 mg of γ-HCH.

C. Contact

As the susceptibility of the different strains was not constant, it had to be estimated at regular intervals during the investigations. So it was of importance to use the very simple contact method. The results of this method are in accordance with those of the application method, as can be seen by comparing fig. 5 and 6. In the experiments presented here, the susceptibility of the 8 strains was estimated about simultaneously by application and by contact. In the contact method 20 flies of each strain were put into a jar, the walls of which were covered with a piece of filter paper impregnated with 1 mg of γ-HCH. Just as in the application experiments the logarithm of time is plotted against the percentage of the flies knocked-down. It follows from these experiments that it is possible to use the results of the contact method in the investigations on the relation between the absorption, detoxication and excretion and the degree of resistance.

1. *Susceptibility to γ-HCH*

In fig. 7 is shown the susceptibility of the strains during the greater part of our study. As the distribution of the degree of resistance in every strain is rather homogeneous, the time needed to give 50 % knock-down (ET 50) can be used as a measure of the degree of resistance. For the comparison with the physiological results these values will be mainly used.

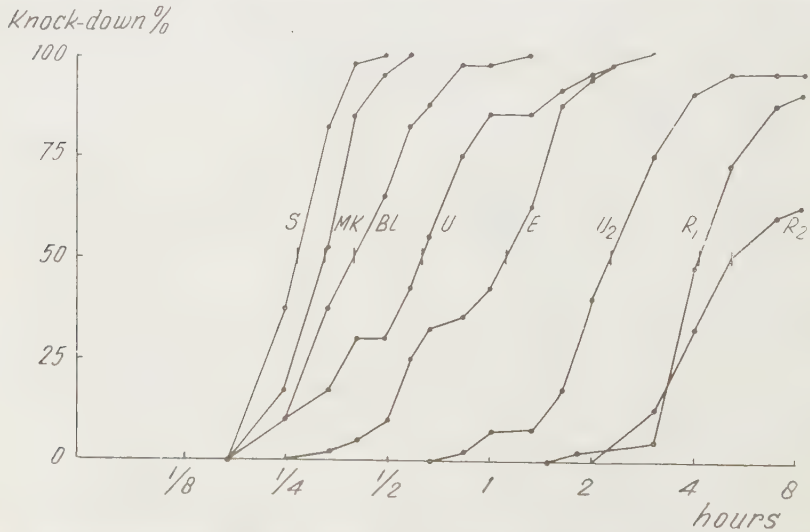

Fig. 7. Knock-down upon contact with a filter paper impregnated with 1 mg of γ-HCH.

As the susceptibilities of some strains were not entirely constant, their changes will be mentioned here. The susceptibility to γ-HCH of

the strains S, Bl, and MK was practically constant. That of the strains U and E was highly variable. Sometimes, contrary to the situation shown in fig. 7, strain U was more resistant than E. Both strains were always intermediate between strain Bl and U_2. The strains U_2, R_1 and

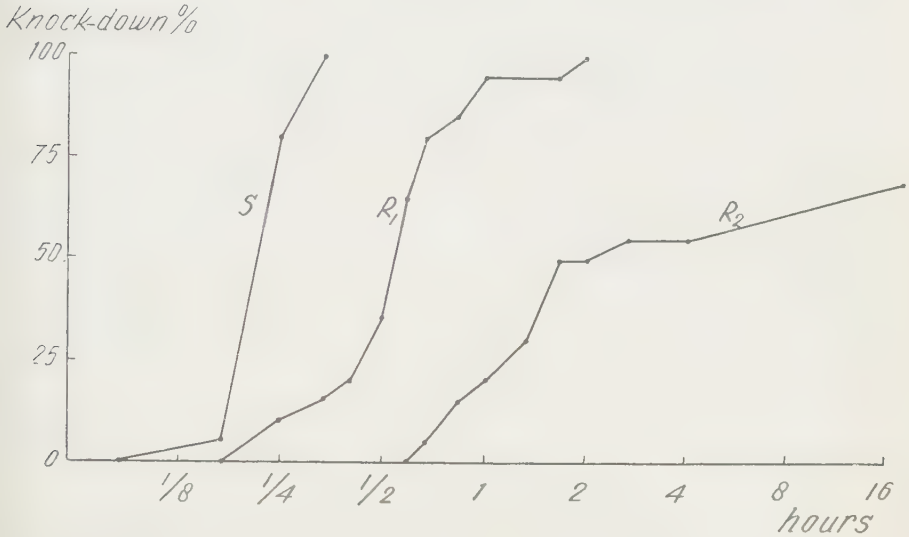

Fig. 8. Knock-down upon contact with a filter paper impregnated with 200 mg of DDT at about 20° C. 1954.

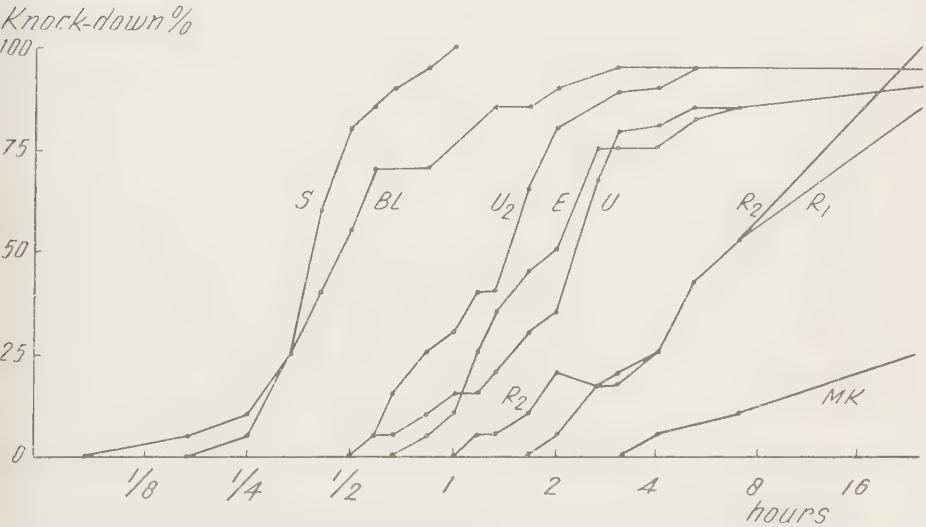

Fig. 9. Knock-down upon contact with a filter paper impregnated with 100 mg of DDT at 25° C. 1955.

R_2 were selected in every generation with a rather low dose of γ-HCH (p. 25). Resistance was only slightly increased by this selection.

2. *Susceptibility to DDT*

The susceptibility of the different strains to DDT was also estimated with the contact method. In fig. 8 and 9 the results of two experiments are shown, one of which was carried out a year before the other. In spite of the fact that the concentrations and temperatures used were not the same, a big difference in susceptibility of strain R_1 is apparent. The difference in susceptibility to DDT between strain R_1 and R_2, caused by the previous selection of R_1 to DDT susceptibility (table 3), disappeared gradually, probably as a result of the selection with γ-HCH. The selection with γ-HCH of U to U_2, however, was not accompanied by an increase in resistance to DDT. This may confirm the results of Barbesgaard and Keiding, who concluded that a gene for resistance to DDT may contribute to the resistance to γ-HCH, although this gene is not indispensable for it (p. 6).

V. METABOLISM OF HCH ISOMERS AFTER INJECTION[1]

METABOLISM OF γ-HCH

From the study of the resistance to DDT it is known that of the three physiological factors which could have an influence on the amount of the toxicant in the insects (p. 15), only the detoxication is of importance to resistance. From the estimation of the susceptibility to γ-HCH with the injection method, it is known that there is an important difference in susceptibility between the strains, although the influence of the absorption has been eliminated. Therefore, the first question to be studied was, whether γ-HCH is metabolized by houseflies and if so, whether there is perhaps a difference in rate of breakdown between susceptible and resistant strains. In the same experiments the importance of the excretion of the toxicant was investigated.

The metabolism of γ-HCH was studied in strain S, R_1 and R_2. Per fly 0.34 μl of an emulsion was injected. In each estimation 70 flies were used, receiving together 22 μg of γ-HCH. They were kept for varying periods of time at 25° C, after which the amount of γ-HCH was estimated with the Schechter-Hornstein method. Three groups of flies were injected with an emulsion without HCH, the mean of the blank values thus found was substracted. Fig. 10 shows the amounts of HCH found in the flies. The decrease with time points to a rapid metabolism or excretion.

[1] partly published as preliminary communications, (Oppenoorth, 1954, 1955).

It might be objected that a decreasing yield of the extraction may have caused these results. This is not probable, however, as the extraction (with boiling acetic acid during 45 minutes) is very vigorous. Moreover, similar results were obtained with another extraction method. In this method the injected flies were ground with sodium-sulfate and extracted with methylenechloride. Although these experiments gave less reproducible results than those in which the flies were directly extracted with acetic acid, the decrease in the amount of γ-HCH with time could be confirmed.

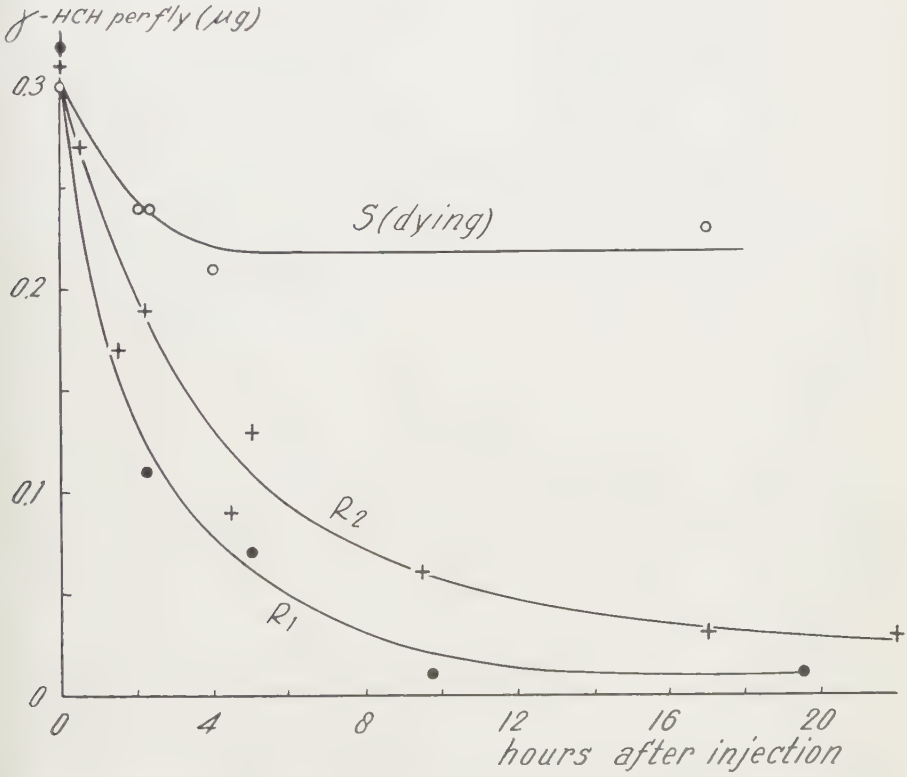

Fig. 10. Amount of γ-HCH plotted against time after injection. 70 flies used for each estimation.

In order to find out whether the decrease in the flies could have been the result of excretion of unchanged HCH the flasks in which they had been kept were rinsed with methylenechloride which was analysed for HCH present. In table 5 it is shown that no γ-HCH was found in the flasks. It follows that the decrease must have been caused by a chemical

conversion. The reaction is stopped if the flies are heated. This was demonstrated by killing 30 minutes after injection two groups of 70 flies in boiling water. In one group, immediately after the flies were heated, 19 μg of γ-HCH was found, in the other 17 μg was present after 22 hours at 25° C. It is probable, therefore, that the conversion of the γ-HCH is enzymatic.

TABLE 5

Amounts of γ-HCH in excreta of groups of 70 flies of strain R_2.

amount injected (μg)	*hours after injection*	*γ-HCH (μg)*	
		in flies	*in flasks*
22	17	2	0
	22	1	0
44	19	4	0

The rate of the breakdown in the three strains is different. This may depend for the greater part on a difference in their reaction to γ-HCH. Strain S shows symptoms soon after the injection and is killed in some hours, whereas strain R_1 and R_2 are not affected. That the breakdown ceases in strain S after four hours may therefore have been caused by its death. Whether the somewhat slower rate in strain S during the first period is caused by the toxic action, or is the result of a smaller breakdown-capacity cannot be concluded. The difference with the resistant strains may as well be a cause as an effect of resistance.

From the results of fig. 10 it becomes probable that the breakdown cannot be the only factor in resistance. The rate of breakdown is higher in strain R_1 than in R_2, whereas R_1 is the more susceptible. Although these differences alone may not be significant, other results pointing in the same direction will be presented later.

It is very difficult to obtain information on the site of the breakdown. A study of this process in isolated organs or tissues would be desirable. However, the large number of insects needed to obtain accurate values in the estimations of HCH precludes any work in which complicated operations are involved. One experiment was carried out by which an impression could be obtained of the site of the breakdown-mechanism. In some groups of 70 flies the abdomina were separated from the rest of the body, 30 minutes after injection of 22 μg of γ-HCH into the thorax. Provided that the parts are kept in a moist atmosphere, they survive for several hours. The anterior parts were capable of normal locomotion even after hours. In some experiments both halves are immediately used for the estimation of γ-HCH, in other cases they are used in the estimation after about 4 hours at 25° C.

In table 6 the results are shown. The amounts found being very near to the limit of the accuracy of the Schechter-Hornstein method, these results still give an indication that breakdown occurs in both halves. Therefore it must take place either in all organs, or in organs that are present in the two parts, *e.g.* in the integument. From table 6 it can also be seen that in the abdomina, representing only 25 % of the body weight, more than half of the toxicant is present 30 minutes after injection. The abdomina contain about 75 % of the body fat, which justifies the supposition that the HCH is deposited in the fat-body.

TABLE 6

Amounts of γ-HCH in abdomina and thoraxes + heads of 70 flies.

Strain	*Part of insect*	*γ-HCH (μg)*		
		30 minutes after injection	*4.40 h. after injection (4.10 h. after separating the parts)*	*converted*
S	Abdomen	9	6	3
	Thorax + head	10	9	1
	Total	19	15	4
R_2	Abdomen	10	4	6
	Thorax + head	8	4	4
	Total	18	8	10

ASSUMPTIONS LEADING TO THE USE OF α- AND δ-HCH

The injection experiments described above are not suited to compare the influence of the breakdown in susceptible and resistant flies. Theoretically there are different possibilities for the study of this problem, if care is taken to investigate the strain under comparable circumstances.

Some possibilities are:

1. the study of the metabolism of γ-HCH at low concentrations, so that the susceptible flies will survive.
2. The inhibition of breakdown and a subsequent study of the difference in susceptibility of the strains.
3. The study of the breakdown *in vitro*.
4. The study of the breakdown-capacity of the strains for non-toxic analogues, if it can be shown that it is determined by the same mechanism as that of the toxicant. The breakdown-capacity can be defined as the rate of breakdown, found in a fly when the amount present at the site of breakdown is not a limiting factor.

In the discussion of the causes of resistance to DDT we have seen

that it is not possible to study the detoxication with amounts that are low enough as not to affect the susceptible flies. This certainly applies to γ-HCH, which is more toxic and can be less accurately estimated.

While in the work on DDT resistance the use of inhibitors and the study of the detoxication *in vitro* were of great help, these techniques are not yet available for γ-HCH. Inhibitors of the breakdown are not known and the techniques for the study of breakdown *in vitro* have not yet been worked out. A first attempt made by us to investigate the breakdown of γ-HCH *in vitro*, in a similar way as used by Kearns for DDT, did not succeed.

In the case of γ-HCH the last possibility can be applied. If it could be shown that the breakdown-capacity of one or more of the non-toxic isomers of HCH is determined by the same mechanism as that of γ-HCH, breakdown in susceptible and resistant strains could be studied under similar circumstances. There are different physiological mechanisms that could determine this capacity. Perhaps, the most plausible assumption is that it depends on the amount of the enzyme in the flies. The working hypothesis would mean in this case that the same enzyme which breaks down the γ-HCH is responsible for the breakdown of one or more of the non-toxic isomers. It should be remarked here, that in the course of the investigations, results were obtained that support the hypothesis. These are discussed in chapter VII.

METABOLISM OF α- AND δ-HCH

The metabolism of α- and δ-HCH was studied after injection in strain S, R_1, and R_2. The β-isomer could not be used, as but too little could be dissolved in the oil of the emulsion.

In fig. 11 and table 7 the results are given of injections of different doses of α-HCH and a high dose of δ-HCH. It appears from the table that a large variation is found in the amounts broken down under the same conditions and in the same strain. This is probably caused by the fact that the properties of the emulsions were less constant than those in the experiments with γ-HCH. For, in order to enable work with smaller numbers of flies per estimation, it was necessary to use more concentrated emulsions. In some experiments with α-HCH these contained even slightly supersaturated oil. Nevertheless the following conclusions seem warranted: α-HCH is rapidly converted, δ-HCH only very slowly. In all cases a more rapid breakdown is found in resistant than in susceptible flies.

The larger breakdown in the resistant strains may be an important factor in resistance but it can also be an occasional difference between the strains. It should be borne in mind in this connexion that strain

TABLE 7

Breakdown of α- and δ-HCH. Thirty flies used for each estimation; horizontal lines represent injections on the same day.

Isomer	*Amount injected per fly (μg)*	*Hours after injection*	*Breakdown (per cent)*		
			S	R_2	R_1
	0.67	4	45	55	65
	0.83	4	36	60	80
α	0.93	4	36	57	57
			34	—	57
			43	—	71
	2.34	5	40	57	—
			23	50	69
			28	43	—
			29	32	—
δ	2.34	18½	25	32	—
			27	46	47
			27	39	37

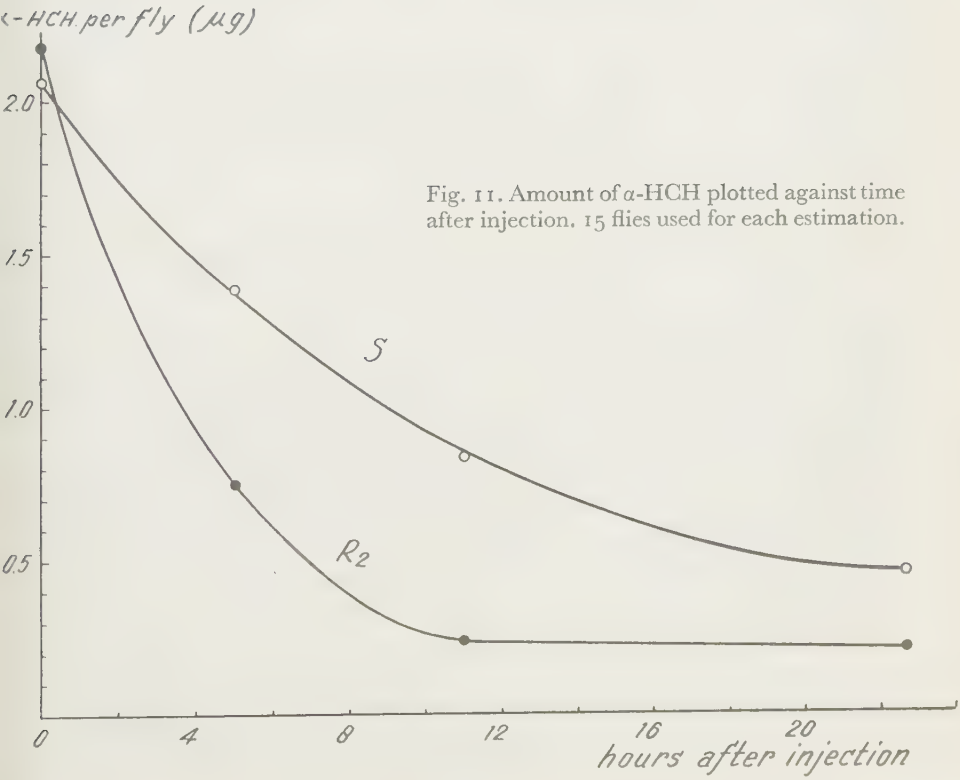

Fig. 11. Amount of α-HCH plotted against time after injection. 15 flies used for each estimation.

R_1 and R_2 are closely related. This means, in fact, that only two genetically quite independent strains have been studied. To get more information on this question therefore more strains have to be drawn into the investigations. As the injection method is rather laborious and has given less reliable results with the α- and δ-isomers, the experiments were carried out with an application method. At the same time this method enables a study of the rate of absorption in different strains.

SUMMARY

1. Injected γ-HCH is metabolized in a susceptible strain S and in two resistant strains R_1 and R_2. This metabolism ceases quickly in strain S, probably as a result of the toxic action.

2. Excretion of γ-HCH does not take place.

3. If it could be shown that the breakdown-capacity of one or more of the non-toxic isomers were determined by the same mechanism as that of γ-HCH, the possible role of detoxication for resistance could be studied.

4. Starting from the assumption in point 3, the metabolism was studied of α- and δ-HCH upon injection in strain S, R_1 and R_2. A higher rate was found in the resistant strains than in the susceptible strain.

5. The larger breakdown-capacity of the resistant strains may be a cause of the resistance, although its importance cannot yet be evaluated, because the difference between the strains could also be occasional.

VI. ABSORPTION AND METABOLISM OF HCH-ISOMERS AFTER APPLICATION

In the preceding chapter a higher breakdown of α- and δ-HCH has been found in the resistant strains R_1 and R_2 than in the susceptible strain S. The study of a larger number of strains is desirable, as this will enable us to decide whether the difference found is only an expression of the normal variation between strains or whether it is correlated with resistance. The investigations were therefore extended to the 8 strains described in chapter III and IV. In these strains the absorption was also studied.

DESIGN OF THE EXPERIMENTS

In groups of 70 flies 0.5 μl per fly was applied of an acetone solution of 0.2 % of α-, γ- or δ-HCH. The groups were kept in flasks at 25° C. After

0, 1½, 3, 6 and 24 hours, respectively, a group was narcotized with CO_2 and washed to remove external HCH. The flasks were rinsed with 2 portions of 15 ml of methylenechloride, which were combined afterwards. In the flies, in the wash and in the flasks HCH was estimated. A group of flies was applied with acetone without HCH, and treated in

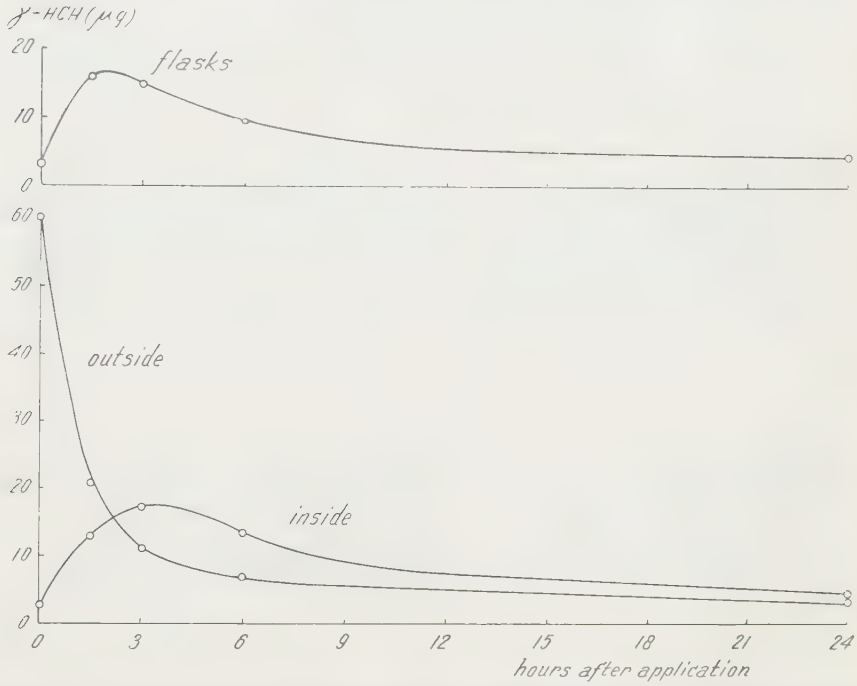

Fig. 12. Amount of γ-HCH in and on 70 flies of strain R_2 and in the flasks in which they had been kept, plotted against time after application of 1 μg per fly.

the same way to obtain blank values. In fig. 12 the results of a typical experiment are given. The curves are obtained by plotting against time after application the amounts found inside, outside and in the flasks.

The curve for *outside* HCH is determined by the rate with which it is absorbed by the flies and yielded to the flasks.

The curve for *inside* HCH is determined by the rate of absorption and breakdown. During the first period the amount increases because the rate of absorption is higher than that of the breakdown. A maximum is reached when the rates of both processes have become equal, followed by a decrease when the rate of breakdown is higher than that of the absorption.

The curve for HCH present in the *flasks* shows how much is lost by the flies. As could be expected this amount depends on the quantity

present on the flies, so that strains with a high rate of absorption yield less than those with a lower rate. The amount on the flasks increases during the first hours, followed by a slow decrease during the later part of the experiment. It is then probably absorbed by the flies directly by contact or as it evaporates from the surface of the flasks and dissolves in the cuticular waxes. The flasks being practically closed it is not probable that HCH is lost by evaporation.

In the flasks only very little HCH is found 24 hours after the application. It can therefore be concluded that no α-, γ- and δ-HCH is excreted by the flies. This corroborates the results with γ-HCH after injection.

To give a better survey of the results, the curves for inside, absorbed and metabolized HCH are given instead of the three curves described above. The amount absorbed can be found by subtracting the sum of HCH on the outside of the flies and on the flasks, from the amount applied. The HCH metabolized is found by subtraction of the total quantity found at a certain time from the amount applied.

RESULTS

With the method described the following application experiments were carried out:

α-HCH on all strains (table 8, fig. 13);
γ-HCH on strain S, U_2 and R_2 (table 9, fig. 14);
δ-HCH on strain S, E, U_2 and R_2 (table 10, fig. 15).

Furthermore with α-HCH a number of experiments was carried out in which the amount was estimated 6 hours after application. This is about the moment that the maximum is found in the flies. The results are shown in table 11 and 12. From these tables it appears that the results of the different experiments vary to a large extent. This is probably due to variations in the animal material and by inaccuracies in the application method. Because of this spread conclusions can only be drawn from a large number of experiments.

As under the present circumstances the β-isomer was practically not absorbed it could not be used in the experiments.

For the absorption, the metabolism and the inside amount of HCH we shall examine successively whether a relation with resistance is present. The investigations have been chiefly carried out with the aid of α-HCH, to a smaller degree with δ-HCH.

Conclusions for the γ-isomer can, of course, only be drawn from the data if the working hypothesis discussed in chapter V is true. This hypothesis must even somewhat be extended. Just as is the case with the

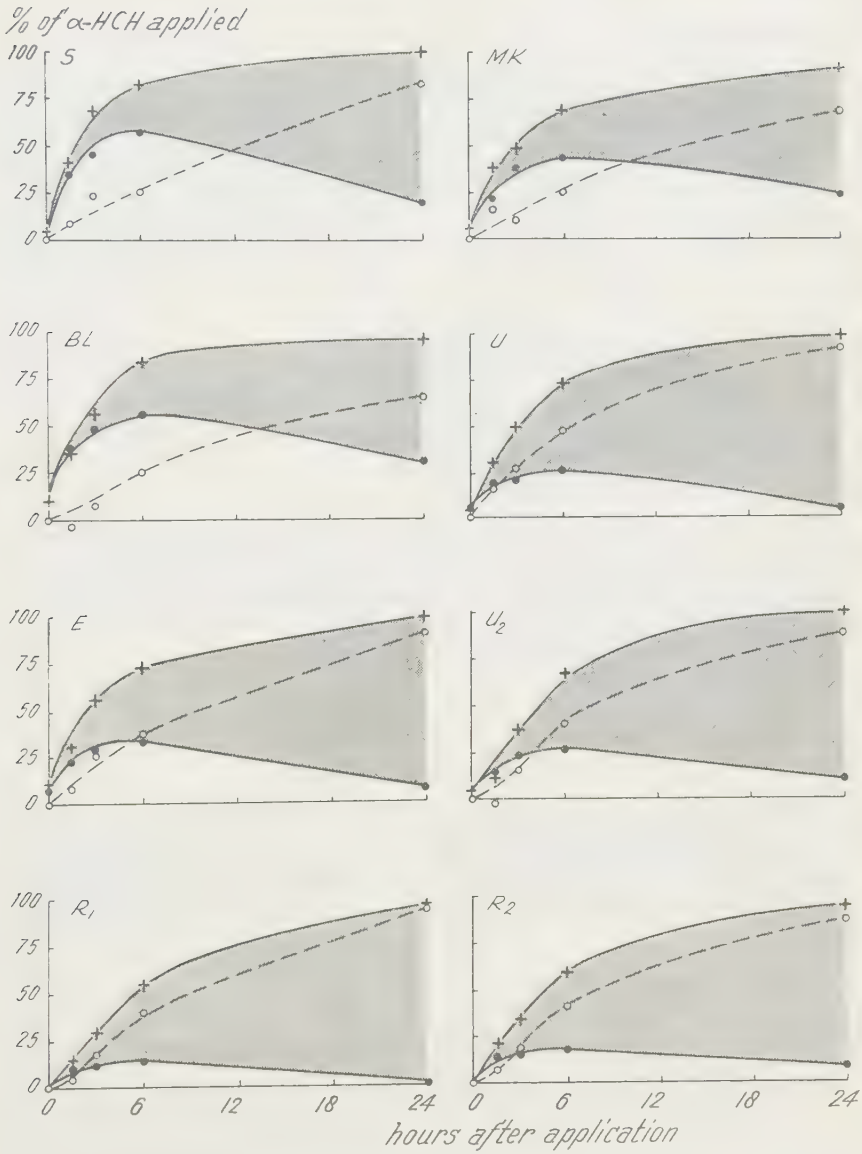

Fig. 13. Percentage of α-HCH applied, plotted against time after application of 1 μg per fly. 70 flies used for each estimation.

+ ——— + HCH absorbed;
• ——— • HCH present in the flies;
○ – – – – ○ HCH converted.

Shaded: effect of conversion.

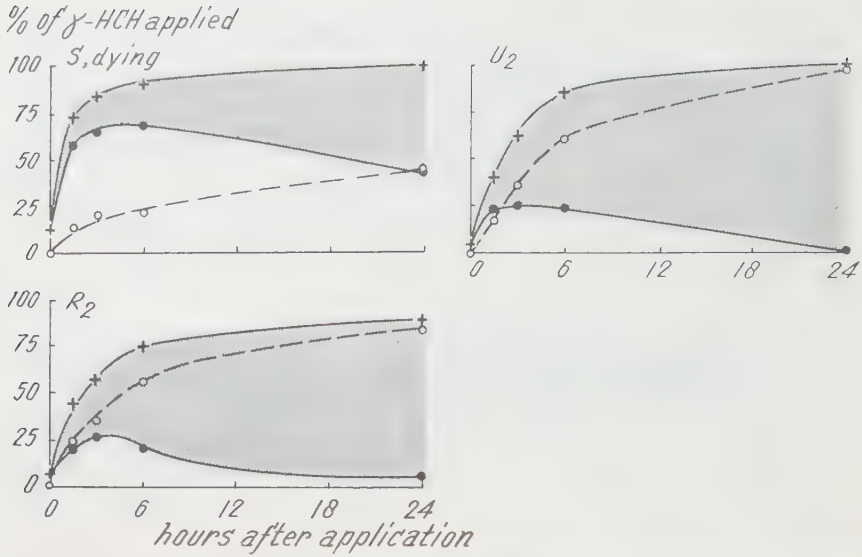

Fig. 14. Percentage of γ-HCH applied, plotted against time after application of 1 μg per fly. 70 flies used for each estimation. For further explanation see fig. 13.

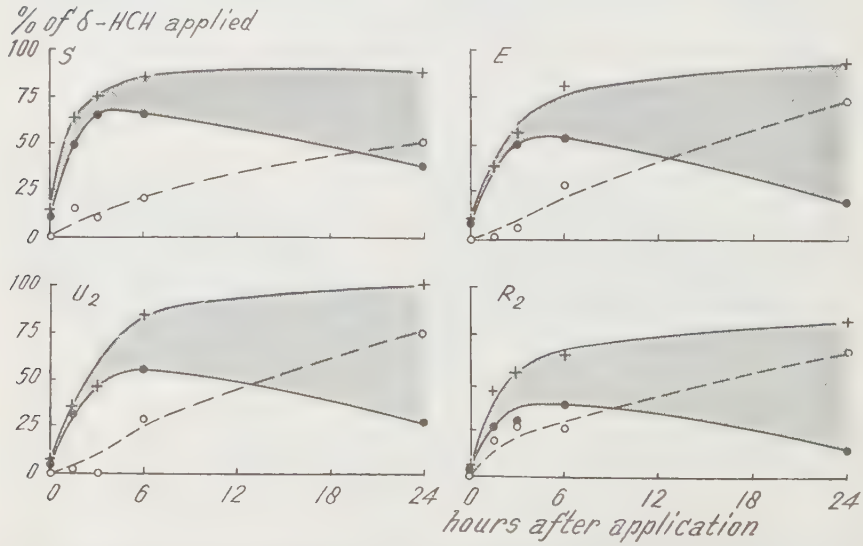

Fig. 15. Percentage of δ-HCH applied, plotted against time after application of 1 μg per fly. 70 flies used for each estimation. For further explanation see fig. 13.

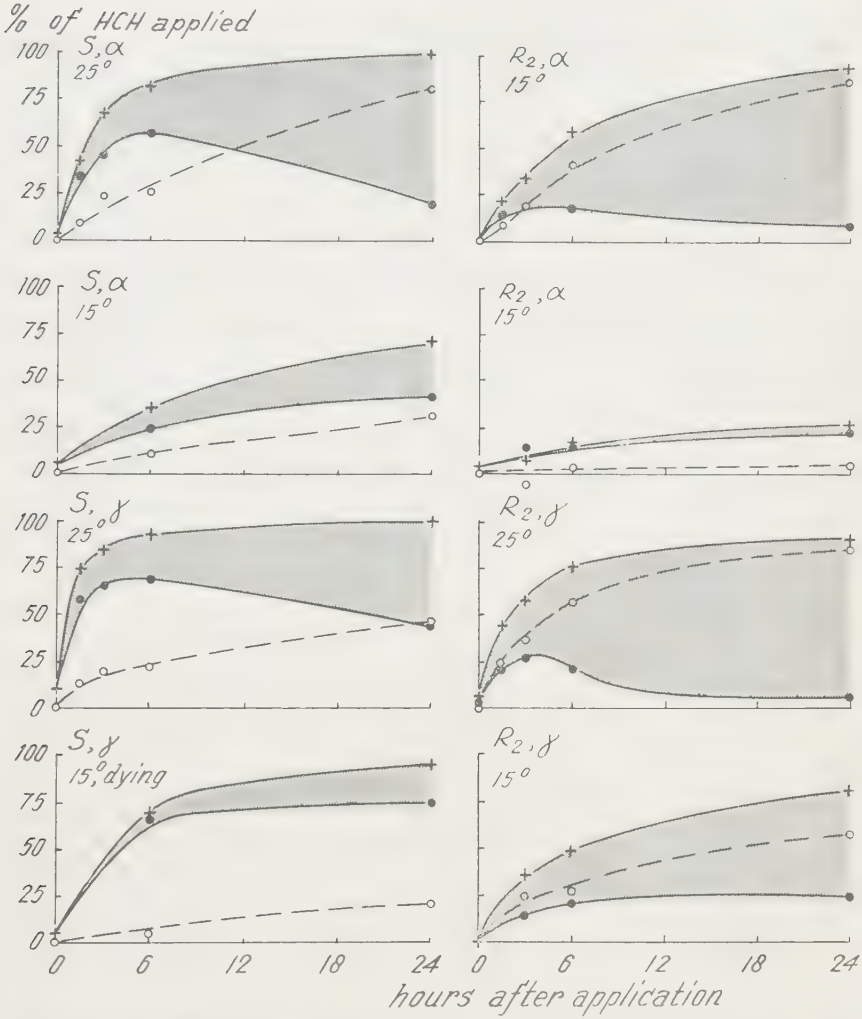

Fig. 16. Percentage of HCH applied, plotted against time at 25° or 15° C after application of 1 μg per fly. 70 flies used for each estimation. For further explanation see fig. 13.

breakdown, the rate of absorption of the three isomers in different strains must also be determined by a common mechanism. It is likely that the ratio of absorption will be about the same in different strains, as the isomers are all fat soluble and the absorption depends probably to a large extent on this property. In chapter VII the results of experiments will be discussed that have a bearing on the hypothesis.

TABLE 8

Per cent of α-HCH at different time-intervals after application of 1 μg per fly.

Strain	Site	Hours after application 0	1½	3	6	24
S	Inside	3	34	45	57	18
	Outside	97	52	32	15	0
	Jars	0	6	0	3	0
	Total	100	92	77	75	18
MK	Inside	5	22	38	43	23
	Outside	94	58	46	24	4
	Jars	1	4	6	7	4
	Total	100	84	90	75	32
Bl	Inside	9	37	48	57	31
	Outside	89	56	36	12	3
	Jars	1	9	9	5	1
	Total	100	103	92	75	35
U	Inside	6	17	21	27	7
	Outside	92	58	39	20	0
	Jars	3	10	11	6	1
	Total	100	84	72	52	8
E	Inside	8	23	29	33	8
	Outside	90	61	37	26	1
	Jars	1	8	7	2	0
	Total	100	92	73	62	10
U_2	Inside	3	14	23	26	11
	Outside	92	76	52	27	0
	Jars	5	14	11	6	0
	Total	100	103	85	59	11
R_1	Inside	1	11	12	14	1
	Outside	98	72	61	37	0
	Jars	1	13	10	8	4
	Total	100	96	83	60	5
R_2	Inside	1	14	16	18	8
	Outside	96	66	52	33	3
	Jars	3	12	14	8	3
	Total	100	92	82	59	14

Deviations in the totals are due to rounding off.

TABLE 9

Percentage of γ-HCH at different time-intervals after application of 1 μg per fly.

Strain	Site	Hours after application 0	1½	3	6	24
S	Inside	12	58	64	68	44
	Outside	88	20	9	3	0
	Jars	0	7	7	7	1
	Total	100	86	80	78	45
U_2	Inside	3	23	25	24	0
	Outside	96	45	23	9	1
	Jars	1	15	15	6	1
	Total	100	83	63	39	3
R_2	Inside	5	20	26	20	6
	Outside	91	32	17	11	5
	Jars	5	24	23	14	6
	Total	100	76	65	44	17

Deviations in the totals are due to rounding off.

TABLE 10

Percentage of δ-HCH at different time-intervals after application of 1 μg per fly.

Strain	Site	Hours after application 0	1½	3	6	24
S	Inside	11	49	65	65	39
	Outside	86	30	18	7	3
	Jars	3	7	8	8	9
	Total	100	85	90	80	51
E	Inside	9	38	50	53	20
	Outside	91	52	32	9	6
	Jars	0	11	12	9	0
	Total	100	100	94	71	26
U_2	Inside	5	31	46	55	27
	Outside	92	59	41	13	0
	Jars	4	9	13	4	0
	Total	100	98	101	72	27
R_2	Inside	3	26	29	38	17
	Outside	91	38	28	16	4
	Jars	7	17	16	20	12
	Total	100	82	73	74	32

Deviations in the totals are due to rounding off.

TABLE 11

Inside α-HCH, in % of amount applied, 6 hours after application of 1 μg per fly.

	S	*MK*	*BL*	*U*	*E*	U_2	R_1	R_2
	48	43	45	20	27	22	15	18
	53	45	51	26	27	25	24	23
	57	48	55	27	33	26	25	25
	61	48	58	28	35	26	29	25
		63		39	38	27		27
		64						29
Means	55	52	52	28	32	25	23	25

TABLE 12

Outside α-HCH, in % of amount applied, 6 hours after application of 1 μg per fly.

	S	*MK*	*BL*	*U*	*E*	U_2	R_1	R_2
	9	16	11	10	10	18	29	24
	10	17	12	17	12	25	33	25
	12	17	13	20	26	27	34	30
	15	18	16	21	26	27	38	33
		20		23	28			36
		24						39
Means	12	19	13	18	20	25	34	31

TABLE 13

Percentage of α- and γ-HCH at different time-intervals at 15° C after application of 1 μg per fly.

Isomer	*strain*	*site*	*Hours after application* 0	3	6	24
α	S	Inside	7		24	41
		Outside	93		66	28
		Jar	0		0	1
		Total	100		90	69
	R_2	Inside	2	13	12	22
		Outside	95	83	72	67
		Jar	3	10	12	7
		Total	100	106	97	95
γ	S	Inside	3		65	74
		Outside	96		23	4
		Jar	1		7	3
		Total	100		95	80
	R_2	Inside	0	13	20	23
		Outside	97	53	43	7
		Jar	3	11	10	13
		Total	100	77	73	43

Deviations in the totals are due to rounding off.

A. Absorption

In fig. 13 to 16 the absorption is represented by the upper curve. Besides, in table 12 is presented the outside amount of α-HCH found 6 hours after application. As the α-HCH in the flasks had not in all cases been estimated, it was not possible to include the absorption in this table. The amount of outside HCH present at this moment is, however, also a measure for the absorption, so that conclusions can still be drawn from this table.

1. Absorption of the different isomers.

The rate of absorption decreases in the order $\gamma > \delta > \alpha$. Especially the absorption of the α-isomer in considerably slower than that of the two other isomers. In strain S for example 50 % is absorbed in 60, 70 and 120 minutes respectively, in strain R_2 in 120, 140 and 290 minutes. As for γ- and α-HCH the absorption was also estimated at 15° C (table 13, fig. 16). It is considerably decreased at this temperature. In strain S 50 % is absorbed of γ- and α-HCH in 4 and 11 hours respectively.

2. Absorption in different strains.

The rate of absorption of the strains can be compared in two ways *e.g.*: 1. by expressing it as the periods of time needed for absorption of a certain percentage of the dosage applied; 2. by expressing it as the percentage absorbed in a certain period.

The data for the two comparisons can be derived from fig. 13, 14 and 15; moreover, for the second comparison the data of table 12 for α-HCH can be consulted. In fig. 17 and 18 the degree of resistance of the strains is plotted against a measure for the rate of absorption of α-HCH. The degree of resistance is plotted as the logarithm of the time, needed to give 50 % knock-down in the contact method (chapter IV, fig. 7). In fig. 17 the percentage of α-HCH that is present on the flies 6 hours after the application has been used as a measure for the absorption. In fig. 18 the time is used in which 50 % of the dose is absorbed. A low percentage or short time thus means a rapid absorption, whereas a high percentage or a long time means a slow absorption.

From both figures it is apparent that in general a higher degree of resistance is connected with a lower rate of absorption. Strain MK, however, is more or less an exception to this rule. This strain has a slower absorption than would be expected from its degree of resistance. It seems possible that this is related to the very high degree of resistance to DDT of this strain. The selection with DDT may have had an influence on the properties of the integument. The fact that MK is still rather susceptible for γ-HCH would then be caused by other physiological factors.

With the other isomers fewer experiments have been carried out. From fig. 14 and 15 it can be seen that the general trend is the same as with α-HCH. The time in minutes needed for the absorption of 50 % is for γ-HCH: S (dying) – 45; U_2 – 120; R_2 – 130; for δ-HCH: S – 54; E – 110; U_2 – 140; R_2 – 140. These results confirm therefore those obtained with α-HCH. From some results of experiments at 15° C the same tendency is apparent (fig. 16, table 13).

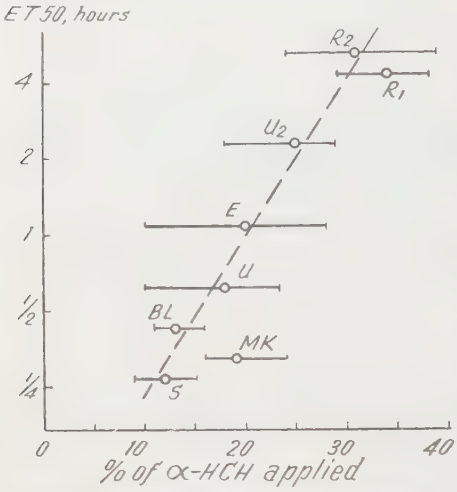

Fig. 17. Degree of resistance of different strains plotted against outside α-HCH in per cents of amount applied, 6 hours after application of 1 μg. Means, lowest and highest values indicated for each strain. Degree of resistance expressed as time needed for 50 % knock-down in the contact method.

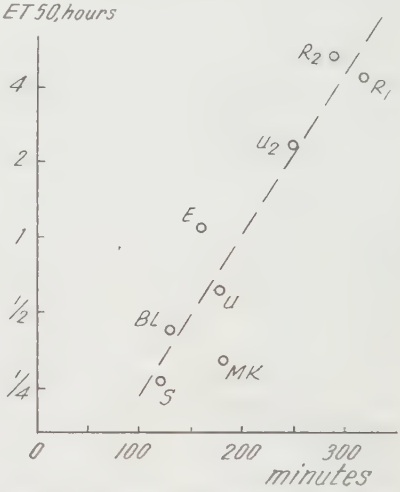

Fig. 18. Degree of resistance (see fig. 17) plotted against time needed for absorption of 50 % of 1 μg of α-HCH in different strains.

It follows that the lower rate of absorption in resistant flies must be regarded as a cause of resistance. The relative importance of this factor is discussed in chapter VIII.

The question arises as to what are the causes of the difference in absorption in the different strains. The finding of an answer is hampered by our scanty knowledge of the absorption process. Probably the solubility in the cuticular lipoids plays an important part. Therefore, the possibility was considered that these lipoids would differ in the strains quantitatively or qualitatively. The following experiments give some support for this supposition.

Some groups of 70 flies of strain S and R_2 were washed with three portions of 10 ml of methylenechloride, before the application of 1 μg

of α-HCH per fly was carried out. The absorption was estimated after 2 hours and compared with that in flies which had not been previously washed (Table 14). It must be pointed out that the washing kills the flies, so that they no longer show cleaning activity or other movements,

TABLE 14

Amount of α-HCH absorbed, in % of amount applied, 2 hours after application of 1 μg per fly.

Strain	*S*	R_2	$S-R_2$
untreated flies	55	27	28
flies washed before application	42	39	3
	43	38	5

while the transport of the HCH in the insects will probably also be changed. These factors certainly would already in itself cause a change in absorption rate, apart from changes of the cuticle caused by an influence of the wash. It is not surprising, therefore, that the rate of absorption is changed by the washing. The essential point, however, is that in these experiments the difference between S and R_2 practically disappears. This may indicate that the lipoids of the cuticle are responsible for the differences in absorption, but there are certainly other possibilities. Experiments on the question of the physiological basis of the differences in absorption are still continued in our laboratory.

B. Metabolism

In fig. 13, 14, and 15 the amount of α-, γ-, and δ-HCH that is metabolized is plotted against the time after application. The rate of the breakdown at every moment is equal to the difference between that of the absorption and that of the changes in the inside amount. These rates are represented in the figures by the tangents of the slopes of the curves. The same relation therefore applies to the tangents of the three curves at a certain moment. In studying the relation between detoxication and resistance the rate of breakdown at a certain moment is of less importance than the breakdown-capacity. The chance that the amount of the toxicant at the sites of breakdown is not a limiting factor, is of course greatest when this amount is as high as possible. This concentration is not known in our experiments, but it will be assumed that it is about proportional to the amount present in the insect as a whole. If this is correct the concentration at the sites of breakdown will be maximal if the amount in the insect is maximal, so that we can best measure the rate at this moment.

Although the rate of breakdown has been measured at the moment

of this maximum, there still remain two possibilities with regard to its relation with the breakdown-capacity.

1. The amounts in the flies, present at this moment, are not a limiting factor for the rate of breakdown. In this case the rate found would be equal to the capacity.

2. The amounts present are still a limiting factor. The rate found in this case would be smaller than the breakdown-capacity.

It may be supposed that in the latter case the rate of breakdown will be about proportional to the concentration present at the site of breakdown and thus with the amount present in the insect. If this is true, a measure for the breakdown-capacity may be obtained when the rate found is divided by the amount present in the insects.

We shall try to decide which of the two possibilities is the most likely, or, in other words, whether the rates of breakdown are dependent on the amounts in the insect. In the cases in which this amount is relatively high, *e.g.* in the experiments with δ-HCH and in the susceptible strains also with the other isomers, there is hardly an indication in the figures that the rate of breakdown is dependent on the amount present. The curve for the breakdown shows little differences in slope. The other cases, the α- and γ-isomers in the resistant strains, show quite another picture. Here is seems that the rate of breakdown is largely dependent on the rate of absorption, which means that the amount present would be a limiting factor.

The results of the calculation of the breakdown-capacities, starting from the two suppositions are presented in table 15. As described above the values of the left column may better apply to the cases of a slow rate of breakdown, whereas those of the right column may be better for the cases of a rapid rate.

TABLE 15

Breakdown-capacity for HCH, as calculated according to two different assumptions

	Breakdown-capacity = rate of breakdown (μg/hour)			Breakdown-capacity = rate of breakdown in μg/hour divided by amount of inside HCH in μg		
Strain	α	γ	δ	α	γ	δ
S	0.033	0.025[1]	0.029	0.06	0.04[1]	0.05
MK	0.041			0.10		
Bl	0.043			0.08		
U	0.052			0.19		
E	0.047		0.033	0.16		0.06
U_2	0.065	0.100	0.042	0.25	0.38	0.08
R_1	0.065			0.46		
R_2	0.062	0.090	0.038	0.33	0.35	0.10

[1] Flies dying during the experiment.

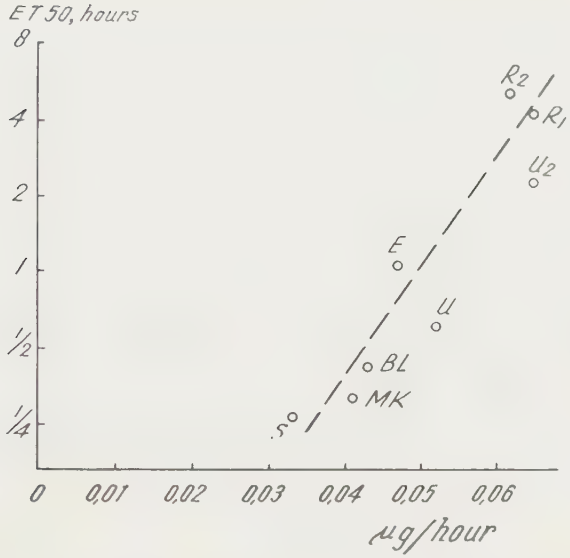

Fig. 19. Degree of resistance of different strains (see fig. 17) plotted against their breakdown-capacity for α-HCH in μg/hour, if breakdown-capacity = rate of breakdown at the moment of the maximum inside amount.

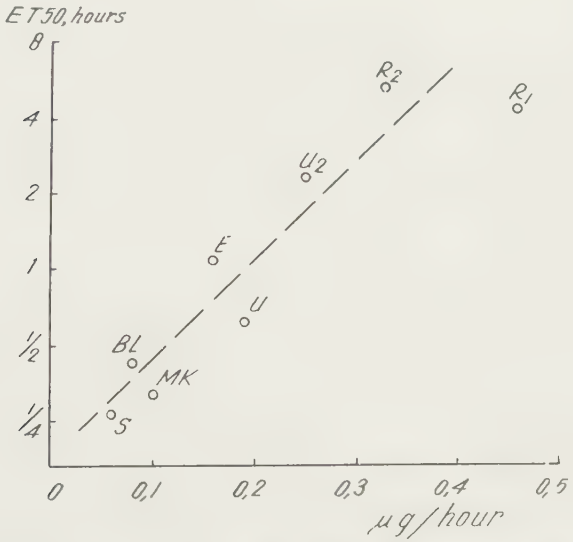

Fig. 20. Degree of resistance of different strains (see fig. 17) plotted against their breakdown-capacity for α-HCH, if breakdown-capacity = rate of breakdown at the moment of the maximum inside amount devided by amount in μg present.

1. Breakdown-capacity for different isomers

The breakdown-capacity decreases in the order $\gamma > \alpha \geqslant \delta$. This order is found in both parts of table 15. The capacity to degrade the δ-isomer is much smaller than that for the α- and γ-isomers, which show only a small difference.

2. Breakdown-capacity in different strains

In general, the breakdown-capacity for α-HCH is higher the more resistant the strains (table 15, fig. 19 and 20). This applies to the values calculated according to the two suppositions. When it was supposed for the calculation that the amount of HCH was a limiting factor, the breakdown-capacity increased with resistance by the greater factor. Whereas the relation between breakdown-capacity and resistance is apparent for α-HCH, it is less clear in the case of δ-HCH.

From the relation found it can be concluded that the breakdown is a cause of resistance. It is difficult to evaluate the significance of this factor. In order to obtain an impression of this significance an attempt was made to stop the breakdown. To this end some experiments were carried out at 15° C. However, as can be seen in fig. 16, the conversion is still apparent at this temperature. Therefore these experiments can give no information on the relative importance of the detoxication. This importance is further discussed in chapter VIII.

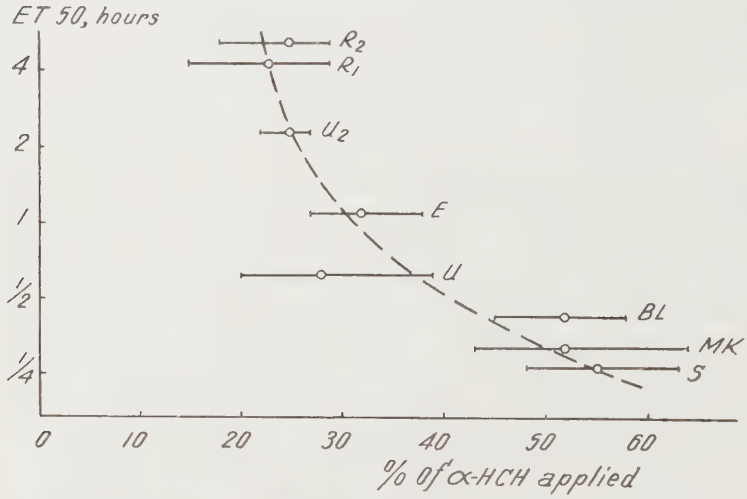

Fig. 21. Degree of resistance of different strains (see fig. 17) plotted against maximum inside α-HCH in percents of amount applied, after application of 1 μg per fly. Means, lowest and highest values indicated.

C. Inside HCH

The amount of HCH present in the insects at a certain moment after application, is the result of the combined influence of the absorption and the conversion of which it has been shown that they are correlated with the resistance. In fig. 13, 14 and 15 the inside amounts found are plotted for the α-, γ-, and δ-isomer.

Moreover, in table 11 for α-HCH more results are shown of estimations 6 hours after the application of 1 μg per fly.

In fig. 21 the maximum amount of α-HCH is plotted against the degree of resistance. In general a lower inside amount is found in strains with higher resistance. This is also apparent from fig. 13, 14, and 15, in which the curves are arranged in the order of increasing resistance.

In fig. 16 it can be seen that also at 15°C the inside amounts of α- and γ-HCH found in strain S are much larger than in strain R_2.

SUMMARY

1. The rate of absorption of the isomers decreases in the order $\gamma > \delta \geqslant \alpha$.

2. The rate of breakdown decreases in the order $\gamma > \alpha \geqslant \delta$.

3. Correlations are found between resistance and the rate of absorption, the breakdown-capacity and the amount of α-HCH found in the flies. With the δ-isomer a smaller number of experiments has been carried out, and the correlations are less clear.

4. According to the assumptions to be proved in chapter VII, therefore, the absorption and detoxication are two factors in the resistance to γ-HCH.

5. The differences in the rate of absorption are probably caused by a qualitative or quantitative difference in the lipoids of the cuticle.

VII. EVIDENCE FOR THE ASSUMPTION MADE IN CHAPTER V

BREAKDOWN-CAPACITY

In chapter VI it was found, that in general a strain has a higher breakdown-capacity to α- and δ-HCH the higher its degree of resistance. This correlation can be explained only if the selection with γ-HCH affects the breakdown-capacity for these substances. It is practically certain that this has been brought about by an increase of the breakpown-capacity for γ-HCH and that this capacity is wholly or partly the same as that responsible for the breakdown of the other isomers. If the mechanism is entirely the same, we must expect not only a correlation with resistance but also an approximately equal factor between

the breakdown-capacity of different strains for each isomer. If the mechanism is only partly the same this factor will be different for the isomers. This would for instance be the case if another breakdown mechanism were present for one of the isomers, independent of the degree of resistance.

The quotient of the breakdown-capacities of the strains S and R_2 for the isomers can be calculated from the data of table 15. For the γ-isomer we may only find minimum values, as the capacity found in strain S may be too small as a result of the mortality in this strain. For the values of the left part of this table we find: $\alpha = 0.53$; $\gamma > 0.28$; $\delta = 0.76$. For the right part we find: $\alpha = 0.18$; $\gamma > 0.11$; $\delta = 0.50$. It appears from these data that the factors in the case of α- and γ-HCH correspond better than for the γ- and δ-isomers. It is therefore probable that the breakdown-capacity of the α- and γ-isomers is for the greater part determined by the same mechanism, whereas the capacity to degrade the δ-isomer may be partly dependent on another mechanism. It follows that the data for the α-isomer give better information on the breakdown-capacity for the γ-isomer than those of the δ-isomer.

ABSORPTION

Just as in the case of the breakdown, on p. 41 a similar assumption has been made for the absorption of the different isomers. It is true that the absorption can be studied under the same conditions in different strains when the flies are dead. It is desirable, however, to study the absorption in living flies, but here the toxic action of the γ-isomer may interfere. The fact that a correlation is found between the rate of absorption of α-HCH and resistance gives in itself already strong support for the correctness of the assumption.

TABLE 16

Time in minutes, in which 50 % is absorbed of a dose of 1 μg of HCH per fly.

				Ratio, if $\alpha = 1$		
Strain	α	γ	δ	α	γ	δ
S	110	40[1]	50	1	0.4[1]	0.5
E	160		110	1		0.7
U_2	260	120	140	1	0.5	0.5
R_2	280	120	140	1	0.4	0.5

[1] Flies dying during the experiment.

In table 16 the periods of time are mentioned needed for absorption of 50 % of a dose of 1 μg per fly in the different strains. Moreover the

ratios between the periods found for the three isomers are calculated for the different strains. It appears that the ratios are about the same in all strains. This lends strong support to our assumption. Therefore conclusions can be drawn on the rate of absorption of the γ-isomer from the results obtained with the α- and δ-isomers.

CONCLUSIONS

The assumption according to which the breakdown-capacity of the α-, γ-, and δ-isomers of HCH is determined by the same mechanism is probably correct. The same is true of the assumption on the rates of absorption of these isomers.

VIII. DISCUSSION

In chapter VI it has been demonstrated that a slower absorption and a higher detoxication are causes of the resistance. We will discuss here the relative importance of these two factors and the question whether it is likely that other physiological differences are involved.

Both factors have an influence on the maximum amount of the toxicant that is reached in the flies. After applying equal amounts of α-HCH onto flies of susceptible and resistant strains we found a ratio of the maxima of about 3 : 1. A factor of this magnitude seems at first sight insufficient to explain the very large difference in resistance (even a factor 20 after injection). It is possible, however, that the differences found have a larger effect on the concentration of the toxicant at the site of action.

This for the insect more favourable situation at the site of action might, for instance, for the greater part be caused by the distribution in the body, by which a large part of the toxicant is stored in relatively unaffected organs, such as the integument and the fat body. Thus the slower absorption and the higher rate of detoxication could be more effective in preventing a critical concentration from reaching the site of action. In chapter V we have seen that there is indeed an indication for the HCH being rapidly taken up by the fat body. It should be borne in mind that qualitative or quantitative differences in the fats are not at all needed for this effect. A similar situation is probably of importance in the case of DDT resistance (p. 13).

Another possibility is that the detoxicating mechanism is especially located favourably with regard to the site of action. We are here on purely speculative ground, however.

Whether the differences found can entirely explain the resistance to

γ-HCH can therefore not be decided at present. There is an indication though, that another factor plays a part. Of the two strains R_1 and R_2, the latter is somewhat more resistant. Both after injection with γ-HCH and in the experiments with α-HCH a larger breakdown-capacity is found in R_1. Moreover R_1 has a somewhat lower rate of absorption than R_2. Although the differences are only small, it is clear that there is a discrepancy here which can only be explained by the influence of another, unknown factor.

We will now examine whether in the scarce literature on HCH there are indications for the existence of other resistance mechanisms. Bradbury *et al.* (1954) administered C^{14}-labeled γ-HCH by a contact method to a susceptible and a resistant strain of flies. The latter had been reared in the presence of non-labeled γ-HCH. The resistant flies picked up less than did the susceptible ones. The authors believe this to have been caused indirectly by the fact that the resistant flies had already γ-HCH (17 μg per gram) in their bodies as a result of the larval treatment. A primary difference in the rate of absorption might, however, also have been the cause, as in our experiments no influence of inside amounts has been found on the rate of absorption. Indeed a higher rate of absorption was found in the susceptible strains although these contained much more inside HCH than resistant flies did.

From the fact that after treatment of the larvae relatively large amounts were present in the pupae (40 μg/g) and in the newly emerged flies (17 μg/g) the authors conclude that the presence of a detoxicating mechanism is unlikely. Yet, it is quite possible, that these amounts are only a small remainder of the quantity taken up by the larvae. Of DDT-resistant flies it is also known that they may contain large amounts although in this case the detoxication is certainly an important factor (p. 13). It cannot be concluded from this study therefore, that the metabolism of γ-HCH is not a factor in resistance, or that another factor must be involved.

In a recent paper (Bradbury and Standen, 1955) the uptake, absorption and detoxication in the same strains were further studied. After a certain period of contact the susceptible strain had accumulated a higher amount of γ-HCH both outside and inside. In the two strains a breakdown-product is formed, in the resistant strain in larger quantities than in the susceptible one. The authors point out that no conclusions on the mechanisms involved in resistance can be drawn from these experiments, because the doses used were lethal to the susceptible strain.

Evidence for the existence of more than one factor in the resistance to γ-HCH can be obtained from the results of a study on the specificity of resistance. By selection with γ-HCH, chlordane or dieldrin, Decker

and BRUCE (1952) obtained strains that were most resistant to the toxicant with which they had been selected and less to the other toxicants. So, in this case, resistance to each toxicant depends partly on specific and partly on aspecific mechanisms. It is probable therefore, that for each toxicant at least two mechanisms are involved.

KEIDING (1953) also comes to the conclusion that resistance to γ-HCH and to chlordane can be largely independent of each other.

Contrary to these findings are those of BUSVINE (1954). He estimated the degree of resistance of four resistant strains and a susceptible one to a number of insecticides of the group of the chlorinated hydrocarbons, *viz.* α- and β-chlordane, isodrin, endrin, aldrin, dieldrin, γ-HCH and DDT. Of the four resistant strains three had been selected with DDT and γ-HCH in the field and one with DDT and chlordane. Nevertheless they all showed a practically constant ratio between their LD's 50 for the different toxicants, with the exception of DDT. This points to a common defence mechanism to the toxicants mentioned.

It is apparent from the literature that there are mechanisms for resistance which are specific and others which are aspecific. Of the two mechanisms in our experiments the differences in absorption are probably aspecific. But it is not likely that this factor is identical with the non-specific mechanism of BUSVINE's study. First the differences in rate of absorption are probably not large enough to explain the high level of resistance of the strains. Second, it is not probable that the resistance caused by the differences in absorption is limited to the group of insecticides for which BUSVINE found cross-resistance. It is for instance to be expected that this difference would also apply to DDT, as the absorption of this substance will probably also be affected by a difference in the lipoids of the cuticle. Yet DDT resistance of the strains of Busvine was to a large extent independent of that to the other substances.

The specificity of the detoxication-mechanism is not known. The two strains E and U are also used in the study of BUSVINE and must therefore possess the mechanism responsible for the cross-resistance. If the detoxication-mechanism for γ-HCH had a low specificity it might explain the cross-resistance. With a bioassay method BUSVINE studied the detoxication after injection of dieldrin and endrin in oil. He found only a slow detoxication for the two toxicants of about the same magnitude, whereas resistance is much higher to dieldrin than to endrin. Busvine concludes from this fact that detoxication is not the mechanism responsible for the cross-resistance. In our opinion it is possible that the rate of detoxication found after injections in oil will be determined by the rate with which the toxicant leaves the oil rather than by the detoxication-capacity of the flies. Thus the breakdown mechanism may still be the aspecific factor involved.

The cross-resistance may also be explained by other mechanisms. Busvine advances some arguments in favour of the idea that this mechanism would be closely related to the mode of action of the toxicants. The compounds for which cross-resistance has been demonstrated would all possess the same toxophore. Resistance would then depend on the ability to escape the action of this toxic configuration. In this case the detoxication mechanisms would play a secondary role only, so that, at least in the 4 strains studied by Busvine, they would not disturb the constant ratio of the LD's 50 to the toxicants.

It is clear that our knowledge of the resistance of Musca is still relatively poor. Only by intensive investigations on the resistance to the many toxicants involved may we hope to enlarge our insight into this intricate problem. These investigations have to go hand in hand with the study of the mode of action since the two problems are closely connected.

Some remarkable data are known on the rates of absorption of the different isomers of HCH. Although it is not yet possible to explain them, a discussion is desirable because they point to an interesting problem that has still to be solved.

The rate of absorption in the flies decreases in the order $\gamma > \delta > \alpha$. The same order was found by Armstrong *et al.* (1951) for the absorption in grain weevils *Calandra granaria* L. which were brought into contact with HCH-impregnated filterpaper. This order of absorption appeared to be another than that found for the solubility of the isomers in the cuticular waxes of the weevils. They calculated this solubility by starting from the assumption that the waxes of the cuticle were saturated when during contact the maximal amount was found on the outside of the weevils. A decrease was found in the order $\delta > \gamma > \alpha$.

Thus the γ-isomer has a far greater absorption rate than would have been the case if the absorption depended on the solubility in the waxy layer. The property of γ-HCH that favours its absorption to such an extent is not known.

The rate of the detoxication of the isomers in the flies decreases in the order $\gamma > \alpha > \delta$. In mice quite another order has been found (Van Asperen, 1950). Upon intravenous injection the γ- and δ-isomers are metabolized at about the same rate, while the rate for α-HCH is much lower. The order of the rates with which the isomers are degraded by alkali to trichlorobenzene is again different namely $\alpha > \delta > \gamma > \beta$ (Kauer *et al.*, 1947). This agrees with the fact that Kearns (1955) found the detoxication product in the flies to be not aromatic and that according to Bradbury and Standen (1955) no trichlorobenzene is formed but probably a water-soluble compound. It follows that the reaction in the flies is different from that by alkali *in vitro*.

IX. SUMMARY

1. The present study deals with the problem of the physiological causes of the resistance to γ-HCH in *Musca domestica* L. Three processes have been studied that might influence the amount of γ-HCH in the flies *viz.* absorption, detoxication, and excretion.

2. The HCH is estimated with the SCHECHTER-HORNSTEIN method. By comparing its results with those of a bioassay method it is demonstrated that only HCH and no conversion-products are estimated.

3. There is a difficulty that makes it impossible to study the three processes with γ-HCH in susceptible and resistant flies under the same conditions. If doses are administered high enough to permit the chemical estimation, the toxic action of γ-HCH results in a rapid kill of the susceptible flies. This can of course strongly influence these processes in the susceptible strains so that it cannot be decided whether differences found with the resistant strains are a result or a cause of resistance.

4. It is assumed that for the different isomers the rate of absorption as well as the rate of the detoxication are each determined by the same mechanism. If this is true, conclusions can be drawn on the rates of these processes for γ-HCH from experiments with the α- and δ-isomers. In this way the difficulty mentioned can be overcome.

5. The absorption is studied after topical application of 1 μg of one of the isomers onto the dorsal thorax.The rate of absorption of α-HCH is estimated in 8 strains, varying from highly susceptible to highly resistant to γ-HCH. A negative correlation is found between the rate of absorption and the degree of resistance of the strains (fig. 17 and 18). The results obtained with δ-HCH show the same tendency. This correlation makes the assumption very probable that the rates of absorption of the three isomers depend on one and the same mechanism, for, if it were not correct, the correlation would have been entirely accidental. The assumption is also supported by the fact that about the same ratio is found between the rates of absorption of the isomers in different strains (table 16). The smaller rate of absorption with increasing resistance will therefore also apply to γ-HCH. It is concluded that *a decreased rate of absorption of γ-HCH is a cause of resistance.*

6. In one susceptible and two resistant strains γ-HCH is injected. A rapid conversion is found in the resistant strains. In the susceptible strain the breakdown is smaller at the beginning and eventually ceases completely (fig. 10). This might be caused by the toxic action on the flies. The α- and δ-isomers are also degraded more slowly in the susceptible than in the resistant strains (fig. 11, table 7). No HCH is found in the excreta.

7. The detoxication in the 8 strains is studied after application of 1

μg per fly. In this way the breakdown-capacity is not estimated, as the breakdown also depends on the rate of absorption. It was tried to account for this difficulty by calculating the breakdown-capacity in two ways. The results thus obtained are presented in table 15. It appears from this table and also from fig. 19 and 20 that a correlation is found between the resistance and the breakdown-capacity for α- and δ-HCH irrespective of the calculation used.

This correlation for the non-toxic isomers can, just as in the case of the absorption, only be explained by assuming that the breakdown-capacities depend on one and the same mechanism. Therefore a correlation must be present between the breakdown-capacity for γ-HCH and the degree of resistance. It follows that *an increased detoxication of γ-HCH is a cause of resistance.*

8. It is difficult to evaluate the importance of the two mechanisms found. They may have a much larger influence on the concentration of the toxicant at the site of action than on the amount in the insect as a whole. There are some indications in the literature and in our own experiments that one or more additional mechanisms also play a part.

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X. REFERENCES

ANDERSON, A. N., R. B. MARCH, and R. L. METCALF, 1954: Inhibition of the succinoxidase system of susceptible and resistant house flies by DDT and related compounds. Ann. Entomol. Soc. of Amer., **47**, 595–602.

ARMSTRONG, G., F. R. BRADBURY, and H. STANDEN, 1951: The penetration of the insect cuticle by isomers of benzene hexachloride. Ann. Appl. Biol., **38**, 555–566.

ASPEREN, K. VAN, and F. J. OPPENOORTH, 1954: Kwantitatieve bepaling en stofwisseling van hexachloorcyclohexaan. Chemisch Weekblad, **50**, 353–356.

ASPEREN, K. VAN, 1954: Onderzoekingen over het werkingsmechanisme van hexachloorcyclohexaan. Med. Landbouwhogeschool, Gent, **19**, 536–545.

BABERS, F. H., 1949: Development of insect resistance to insecticides. U.S. Dept. Agr. Bur. Entomol. Plant Quarantine E–776, 1–31.

BABERS, F. H., 1953: Chemical control and resistance to insecticides by agricultural pests. J. Econ. Entomol., **46**, 869–873.

BABERS, F. H., and J. J. PRATT, JR., 1951: Development of insect resistance to insecticides-II. U.S. Dept. Agr. Bur. Entomol. Plant Quarantine E-818, 1–45.

BABERS, F. H., and J. J. PRATT, JR., 1953: Resistance of insects to insecticides: the metabolism of injected DDT. J. Econ. Entomol., **46**, 977–982.

BABERS, F. H., and C. C. ROAN, 1953: The dehydrochlorination of DDT by resistant cockroaches. J. Econ. Entomol., **46**, 1105.

BARBESGAARD, P., and J. KEIDING, 1955: Crossing experiments with insecticide-resistant house flies. Vidensk. Medd. fra Dansk naturh. Foren., **117**, 84–116.

BIGELOW, R. S., and E. J. LEROUX, 1954: Distinct morphological differences between DDT-resistant and non DDT-resistant strains of the housefly, *Musca domestica* L. The Canadian Entomologist, **86**, 78–86.

BRADBURY, F. R., and H. STANDEN, 1954: The colorimetric determination of benzene hexachloride in insect tissues. J. Science Food and Agriculture, **5**, 252–256.

BRADBURY, F. R., and H. STANDEN, 1955: The fate of γ-benzene hexachloride in normal and resistant houseflies. I. J. Science Food and Agriculture, **6**, 90–99.

BRADBURY, F. R., P. NIELD, and J. F. NEWMAN, 1953: Amount of gamma-benzene hexachloride picked up by resistant houseflies bred on a medium containing benzene hexachloride. Nature, **172**, 1052.

BRUCE, W. N., and G. C. DECKER, 1950: House fly tolerance for insecticides. Soap and Sanitary Chemicals, **26**, 122–125, 145–147.

BUSVINE, J. R., 1951: Mechanism of resistance to insecticide in houseflies. Nature, **168**, 193–195.

BUSVINE, J. R., 1953a: Laboratory investigation with insecticide-resistant houseflies. Trans. 9th Int. Congr. Entomol. Amsterdam, **2**, 335–339.

BUSVINE, J. R., 1953b: Forms of insecticide-resistance in houseflies and body lice. Nature, **171**, 118–122.

BUSVINE, J. R., 1954a: Houseflies resistant to a group of chlorinated hydrocarbon insecticides. Nature, **174**, 783–785.

BUSVINE, J. R., 1954b: The development of resistance to insecticides. Report 6th Commonwealth. Entomol. Conf.; Commonwealth. Inst. Entomol., London, 60–65.

BUSVINE, J. R., and N. H. KHAN, 1955: Inheritance of BHC-resistance in the housefly. Trans. Royal. Soc. Trop. Med. Hyg., **49**, 455–459.

CHADWICK, L. E., 1952: The current status of physiological studies on DDT-resistance. Am. J. Trop. Med. Hyg., **1**, 404–411.

CHADWICK, L. E., 1955: Physiological aspects of insect resistance to insecticides. In: Origins of resistance to toxic agents, M. G. SEVAG, R. D. REID and O. E. REYNOLDS, editors. Academic Press, 133–147.

CHANG, S. C., and H. H. CROWELL, 1953: Effect of successive treatments of DDT on individual susceptibility in the American cockroach. J. Econ. Entomol., **46**, 467–472.

DECKER, G. C., and W. N. BRUCE, 1952: House fly resistance to chemicals. Am. J. Trop. Med. and Hyg., **1**, 395–403.

DRESDEN, D., 1949: Physiological investigations into the action of DDT. Thesis G. W. v. d. Wiel & Co., Arnhem.

EBBINGE WUBBEN, G. J. H., en F. J. KUIPER, 1947: Het optreden van resistentie tegen chemische bestrijdingmiddelen bij insecten. Rapport T 1186. Alg. Techn. Afd. T.N.O., 1–38.

FISHER, R. W., 1952: The importance of the locus of application on the effectiveness of DDT for the house fly, *Musca domestica* L. Can. J. Zool., **30**, 254–266.

FLETCHER, T. E., 1952: DDT metabolism in two DDT-resistant strains and one non-resistant strain of *Musca domestica*. Trans. Roy. Soc. Trop. Med. Hyg., **46**, 6.

FRITSCH, H., 1952: Versuche zur Analyse des Angriffspunktes kontact-insektizider Stoffe. Biol. Zentralbl., **71**, 512–528.

FRITSCH, H., and H. KRUPP, 1952: Wirkung von Insektiziden auf ein isoliertes Ganglien-Muskel-Präparat von *Dytiscus marginalis* (Gelbrandkäfer). Arch. Exp. Path. Pharmakol., **214**, 227–241.

FULLMER, O. H., and W. M. HOSKINS, 1951: Effects of DDT upon the respiration of susceptible and resistant house flies. J. Econ. Entomol., **44**, 858–870.

GRAYSON, J. McD., 1951: Response of the German cockroach to sublethal concentrations of DDT and benzene hexachloride. J. Econ. Entomol., **44**, 315–317.

HARRISON, C. M., 1951: Inheritance of resistance to DDT in the housefly *Musca domestica* L. Nature, **167**, 855–856.

HARRISON, C. M., 1952: The resistance of insects to insecticides. Trans. Roy. Soc. Trop. Med. Hyg., **46**, 255–261.

HARRISON, C. M., 1953: DDT-resistance and its inheritance in the housefly. J. Econ. Entomol., **46**, 528–530.

HESS, A. D., 1952: The significance of insecticide resistance in vector control programs. Am. J. Trop. Med. Hyg., **1**, 371–388.

HESS, A. D., 1953: Current status of insecticide resistance in insects of public health importance. Am. J. Trop. Med. Hyg., **2**, 311–317.

HORNSTEIN, I., 1952: Determination of technical benzene hexachloride in peanuts and soils. Anal. Chem., **24**, 1036–1037.

HOSKINS, W. M., and P. S. MESSENGER, 1950: Microbioassay of insecticide residues in plant and animal tissues. Advances in Chem. Series, **1**, 93–98.

HOSKINS, W. M., J. M. WITT and W. R. ERWIN, 1952: Bioassay of 1, 2, 3, 4, 5, 6-hexachlorocyclohexane (Lindane). Anal. Chem., **24**, 555–560.

JOHNSTON, C. D., 1951: The *in vitro* effect of DDT and related compounds on the succinoxidase system of rat heart. Arch. Bioch. Biophys., **31**, 375–382.

KAUER, K. C., R. B. DUVALL, and F. N. ALQUIST, 1947: Epsilon isomer of 1, 2, 3, 4, 5, 6-hexachlorocyclohexane. Industr. Eng. Chem., **39**, 1335–1338.

KEARNS, C. W., 1955: The enzymatic detoxication of DDT. In: Origins of resistance to toxic agents, M. G. SEVAG, R. D. REID and O. E. REYNOLDS, editors. Academic Press, 148–159.

KEIDING, J., 1953: Development of resistance in the field and studies of inheritance. Trans. 9th Int. Congr. Entomol., **2**, 340–345.

LALONDE, D. I. V., and A. W. A. BROWN, 1954: The effect of insecticides on the action potentials of insect nerve. Canad. J. Zool., **32**, 74–81.

LEROUX, E. J., and F. O. MORRISON, 1953: Dosage of DDT needed to kill a house fly. J. Econ. Entomol., **46**, 1109–1110.

LeRoux, E. J., and F. O. Morrison, 1954: The absorption, distribution and site of action of DDT in DDT-resistant and DDT-susceptible house flies using carbon[14] labeled DDT. J. Econ. Entomol., **47**, 1058–1066.

Linderstrøm-Lang, K., and H. Holter, 1940: Die enzymatische Histochemie, in Bamann, E., and K. Myrbäck, Die Methoden der Fermentforschung. Thieme, Leipzig, 1132–1162.

Maelzer, D. A., and R. L. Kirk, 1953: A preliminary study of the genetics of DDT-resistance in houseflies. Austral. J. Biol. Sci., **6**, 244–256.

March, R. B., R. L. Metcalf, and L. L. Lewallen, 1952: Synergists for DDT against insecticide-resistant houseflies. J. Econ. Entomol., **45**, 851–860.

Metcalf, R. L., 1955a: Organic insecticides. Interscience publishers N.Y., London.

Metcalf, R. L., 1955b: Physiological basis for insect resistance to insecticides. Physiol. Revs., **35**, 197–232.

Milani, R., 1954: Genetic aspects of the development of resistance to chemical insecticides. 1e Symposium Int. sulla lotta contro gli insetti vettori di malattie transmissibili, 253–274.

Moorefield, H. H., and C. W. Kearns, 1955: Mechanism of action of certain synergists for DDT against resistant house flies. J. Econ. Entomol., **48**, 403–406.

Morrison, F. O., and E. J. LeRoux, 1954: House fly head as site of lethal action of DDT. Can. J. Agricult. Sci., **34**, 316–317.

Newman, J. F., 1953: Resistance of flies to different insecticides. Trans. 9th Int. Congr. Entomol. Amsterdam, **2**, 331–334.

Norton, R. J., 1953: Inheritance of DDT tolerance in the house fly. Contrib. Boyce Thomp. Inst., **17**, 105–126.

Oppenoorth, F. J., 1954: Metabolism of gamma-benzene hexachloride in susceptible and resistant house flies. Nature, **173**, 1000–1001.

Oppenoorth, F. J., 1955: Differences between rates of metabolism of benzene hexachloride in resistant and susceptible house flies. Nature, **175**, 124.

Perry, A. S., and W. M. Hoskins, 1950: The detoxification of DDT by resistant house flies and inhibition of this process by piperonyl cyclonene. Science, **111**, 600–601

Perry, A. S., and W. M. Hoskins, 1951a: Synergistic action with DDT toward resistant house flies. J. Econ. Entomol., **44**, 839–850.

Perry, A. S., and W. M. Hoskins, 1951b: Detoxication of DDT as a factor in the resistance of house flies. J. Econ. Entomol., **44**, 850–858.

Perry, A. S., and B. Sacktor, 1955: Detoxification of DDT in relation to cytochrome oxidase activity in resistant and susceptible houseflies. Ann. Entom. Soc., **48**, 329–333.

Perry, A. S., R. W. Fay, and A. J. Buckner, 1953: Dehydrochlorination as a measure of DDT resistance in house flies. J. Econ. Entomol., **46**, 972–976.

Perry, A. S., J. A. Jensen, and G. W. Pearce, 1955: Colorimetric and radiometric determinations of DDT and its metabolites in resistant houseflies. Agricult. and Food Chem., **3**, 1008–1011.

Perry, A. S., A. M. Mattson, and A. J. Buckner, 1953: The mechanism of synergistic action of DMC with DDT against resistant house flies. Biol. Bull., **104**. 426–438.

Phillips, W. F., 1952: Colorimetric estimation of residual benzene hexachloride Anal. Chem., **24**, 1976–1979.

Pradham, S., M. R. Nair, and S. Krishnaswami, 1952: Lipoid solubility as a factor in the toxicity of contact insecticides. Nature, **170**, 619–620.

Pratt, J. J. Jr., and F. H. Babers, 1953a: Sensitivity to DDT of nerve ganglia of susceptible and resistant house flies. J. Econ. Entomol., **46**, 700–702.

Pratt, J. J. Jr., and F. H. Babers, 1953b: The resistance of insects to insecticides. Some differences between strains of house flies. J. Econ. Entomol., **46**, 864–869.

Reith, J. F., 1953: Het aantonen van het insecticide hexachloorcyclohexaan in groenten. Chemisch Weekblad, **49**, 689–692.

Roeder, K. D., and E. A. Weiant, 1948: The effect of DDT on sensory and motor structures in the cockroach leg. J. Cell. & Comp. Physiol., **32**, 175–186.

Sacktor, B., 1950: A comparison of the cytochrome oxidase activity of two strains of house flies. J. Econ. Entomol., **43**, 832–838.

Sacktor, B., 1951: Some aspects of respiratory metabolism during metamorphosis of normal and DDT-resistant house flies, *Musca domestica* L. Biol. Bull., **100**, 229–243.

Schechter, M. S., and I. Hornstein, 1952: Colorimetric determination of benzene hexachloride. Anal. Chem., **24**, 544–548.

Sherman, M., and L. B. Norton, 1948: The bioassay of gamma benzene hexachloride. J. Econ. Entomol., **41**, 288–292.

Smith, H. S., 1941: Racial segregation in insect populations and its significance in applied entomology. J. Econ. Entomol., **34**, 1–13.

Smyth, T., and C. C. Roys, 1955: Chemoreception in insects and the action of DDT. Biol. Bull., **108**, 66–76.

Sternburg, J., and C. W. Kearns, 1950: Degradation of DDT by resistant and susceptible strains of houseflies. Ann. Ent. Soc. Am., **43**, 444–458.

Sternburg, J., and C. W. Kearns, 1952: Metabolic fate of DDT when applied to certain naturally tolerant insects. J. Econ. Entomol., **45**, 497–505.

Sternburg, J., C. W. Kearns, and W. N. Bruce, 1950: Absorption and metabolism of DDT by resistant and susceptible houseflies. J. Econ. Entomol., **43**, 214–219.

Sternburg, J., E. B. Vinson, and C. W. Kearns, 1953: Enzymatic dehydrochlorination of DDT by resistant flies. J. Econ. Entomol., **46**, 513–515.

Sternburg, J., C. W. Kearns, and H. Moorefield, 1954: DDT dehydrohalogenase, an enzyme found in DDT resistant flies. J. Agricult. Food Chem., **2**, 1125–1130.

Tahori, A. S., and W. M. Hoskins, 1953: The absorption, distribution and metabolism of DDT in DDT-resistant house flies. J. Econ. Entomol., **46**, 302–306, 829–837.

Tattersfield, F., and J. R. Kerridge, 1955: The effect of repeated spraying of insects on their resistance to insecticides. Ann. appl. Biol., **43**, 630–644.

Terriere, L. C., and R. D. Schonbrod, 1955: The excretion of a radioactive metabolite by house flies treated with carbon 14 labelled DDT. J. Econ. Entomol., **48**, 736–739.

Tiel, N. van, 1955: Influence of resins on crystal size and toxicity of insecticidal residues. Thesis–Excelsior, 's-Gravenhage.

Vinson, E. B., and C. W. Kearns, 1952: Temperature and the action of DDT on the American roach. J. Econ. Entomol., **45**, 484–496.

Wiesmann, R., 1947: Untersuchungen über das physiologische Verhalten von *Musca domestica* L. verschiedener Provenienzen. Mitt. Schweiz. Entomol. Ges., **20**, 484–504.

Wiesmann, R., 1954: Der heutige Stand des Insektizid-Resistenzproblems. Mitt. Biol. Bundesanstalt Land und Forstwirtschaft, **83**, 17–37.

Wiesmann, R., 1955: Vergleichende Untersuchungen über die Reitzleitung normalsensibler und resistenter Imagines von *Musca domestica* L. unter der Einwirkung von DDT-Substanz. Mitt. Schweiz. Entomol. Ges., **28**, 251–273.

Winteringham, F. P. W., and J. M. Barnes, 1955: Comparative response of insects and mammals to certain halogenated hydrocarbons used as insecticides. Physiol. Rev., **35**, 701–739.

Winteringham, F. P. W., P. M. Loveday, and A. Harrison, 1951: Resistance of house flies to DDT. Nature, **167**, 106–107.

POPULATION STATISTICS OF THE BAT MYOTIS MYSTACINUS, BASED ON THE MARKING-RECAPTURE METHOD

by

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I. INTRODUCTION

Bat banding in the Netherlands was started in 1936 by BELS (1952). On going out to Singapore in 1952 he left to the authors not only his extensive thesis on the subject, but also his still more extensive basic notes concerning these investigations. After his departure the authors have continued his work, using the same techniques in the same hibernation quarters (VAN HEERDT and SLUITER, 1953, 1954, 1956).

From 1936 till 1951 over 17,000 bats belonging to 14 species had been ringed by BELS, and we were able to add some 4,000 to this number. At this moment the numbers of recaptures have increased sufficiently to allow a quantitative study of the population of *Myotis mystacinus*. This is the most common species found in our main bat haunts, the limestone caves in the province of Limburg, where they have been ringed from 1942 onwards. As age determination of adult specimens is not feasible in the field, we had to base this study on the capture-recapture method, using for the greater part data collected by BELS which, however, were not treated statistically by him.

The main purpose of our investigation is to make estimates of survival rates. From these, other population characteristics, such as age structure, life span, mean longevity and juvenile mortality, can be calculated if the sex ratio in the population as a whole and the reproductive rate of the species are known, and if some additional assumptions are made. We are well aware of the fact that life tables prepared from these survival rates, only provide an introduction to the ecological problems involved: which factors control life and death of bats, and to which extent can each factor be held responsible?

The first part of this paper deals with our sampling technique and the numbers of bats ringed and recaptured. A method to calculate population parameters from these basic data will be given in the chapter "Mathematical considerations". Finally, results of the calculations for the bat species mentioned above are shown and discussed.

II. SAMPLING TECHNIQUE AND DEFINITION OF THE POPULATION

The hibernating quarters where the bats are ringed and recaptured at the end of December or in the beginning of January are located near Maestricht, in the extreme southern part of the Netherlands (fig. 1). They consist of caves in a patch of Cretacious limestone covering approximately 50 square miles (fig. 1). From ancient times till now the soft limestone has been excavated for building purposes, resulting in

the formation of huge labyrinths of galleries with heights varying from 6 to 40 feet and with only pillars left to sustain the ceiling. Each labyrinth is called "a cave".

The bats hibernating in these artificial caves are dispersed over a total passage length of more than a hundred miles. They sleep hanging on the walls and the ceilings of the corridors, mostly separately, but sometimes in small clusters containing 2–5 specimens. Many of them are hiding in crevices and it is often very difficult to find them there.

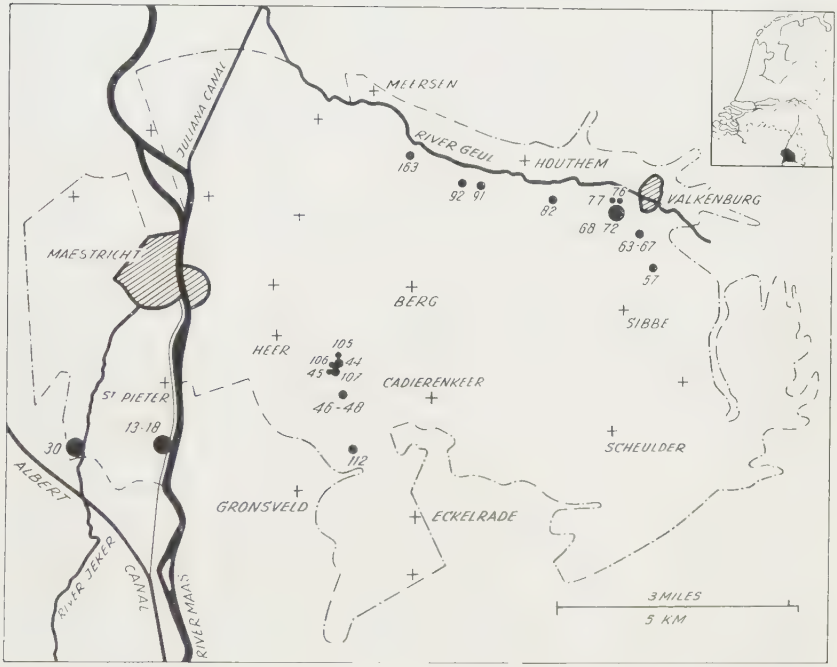

Fig. 1. The cave area of Southern Limburg. The entrances of the sampling caves (mentioned on page 65) are indicated by black dots. The size of a dot is roughly proportionate to the size of the cave involved.

Bels (1952) registered more than 150 cave entrances in the area. The caves vary in size from one short corridor to extensive labyrinths in which one can walk for hours without returning to the same spot. Although the area has been surveyed regularly, it appeared to be impossible to visit all the caves each winter. We had to select a set of 18 caves for sampling. They will be related to as "sampling caves". According to the registration of Bels (1952) the caves which we selected for sampling have the following entrance numbers: 13–18, 30, 44, 45, 46–48, 57, 63–67, 68–72, 76, 77, 82, 91, 92, 105, 106, 107, 112 and 163.

They are dispersed over the area (fig. 1), and as they cover the whole scala of cave sizes they represent most of the available types of caves.

Some of the caves were visited by BELS several times during one winter, but our calculations are based on the results of the first visit in each winter only.

It must be realized that bats may freely shift from a sampling cave to a non-sampling cave, and vice versa. From this possibility the question arises which individuals belong to the population investigated here and which do not. As belonging to our population we consider all those bats which have hibernated at least once in a sampling cave during the period of investigation. They may be divided into two categories:

a) Those which have hibernated once in a sampling cave but did not come back to one during the period of investigation and therefore have not been recapturable. These are animals that die or emigrate (cf. page 78).

b) Those which have hibernated in a sampling cave at least twice during the period of investigation and therefore have been recapturable.

A sampling cave is inspected superficially: only the animals visible at first sight are collected. Probably more bats could have been found by closer examination of the place. But, during each visit approximately the same number of collectors spend about the same length of time on the inspection of a cave. Therefore it may be said that the catching effort at each visit has remained constant, a necessary condition to make the variation in the rate of capture as small as possible.

The bats in the samples are all full-grown specimens of unknown age. Shortly after collection an aluminium ring is fixed around the bat's right underarm, and they are set free again in the same cave. The rings have different numbers and the inscription: ZOOL. MUS. UTRECHT HOLLAND. By means of these rings the bats are recognizable individually.

III. SIZE OF SAMPLES AND NUMBERS OF BATS RECAPTURED

The annual sample sizes are obtained by adding the numbers of bats collected in the sampling caves. The mean annual sample size from 1942–1956 is 162 individuals of *M. mystacinus*. Each sample, except the first, contains bats captured for the first time and bats recaptured. In Table 1 numbers of recaptures of the males of *M. mystacinus* are recorded. This table is constructed in the same way as has been done by BELS (1952) in his table 3. For instance, in the winter 1943/44 (second line) 6 bats were recaptured which were ringed in the winter 42/43; in

the winter 1944/45 (third line) 3 bats were recaptured which were ringed in 42/43, and 5 bats which were ringed in 43/44, etc. The winter 1948/49 has been left out, as the cave area was not visited then.

TABLE 1

Numbers of captured and recaptured males of *M. mystacinus*, 1942–1956 (sampling caves combined).

Winter	42/43	43/44	44/45	45/46	46/47	47/48	48/49	49/50	50/51	51/52	52/53	53/54	54/55	*Total found*
1942/43														69
43/44	6													73
44/45	3	5												58
45/46	4	6	3											90
46/47	1	4	3	8										87
47/48	3	5	3	8	14									112
48/49	—	—	—	—	—	—								—
49/50	1	0	2	5	8	14	—							135
50/51	3	2	0	7	3	9	—	13						106
51/52	1	3	3	3	5	14	—	15	14					121
52/53	2	1	1	4	2	8	—	8	10	24				130
53/54	1	0	2	3	0	5	—	4	4	10	10			90
54/55	0	2	3	3	1	4	—	7	8	16	14	16		113
55/56	0	1	1	2	2	4	—	7	5	7	9	11	20	104

The same individual may be recaptured in several winters subsequently. If, for instance, a bat is ringed in 42/43 and recaptured in the winter of 43/44, 44/45 and 47/48, the recaptures of this individual have been entered in the table as follows: first as those of a bat ringed in 42/43 and recaptured in 43/44, 44/45 and 47/48; secondly as those of a bat ringed in 43/44 and recaptured in 44/45 and 47/48; finally as a bat ringed in 44/45 and recaptured in 47/48.

In Table 2 (third and sixth column) the total numbers of bats recaptured after 1, 2 and subsequent years after ringing, are shown (cf. Bels, 1952, Table 4–18). The figure 133 for "recaptured males after one year", for instance, is obtained by adding the numbers of individuals ringed in 42/43 and recaptured in 43/44 to those ringed in 43/44 and recaptured in 44/45 and so on: $6 + 5 + \ldots + 20 = 133$ (cf Table 1).

In the second and fifth column are given initial numbers, being the

totals of previously captured bats which could have been recaptured after a certain number of years, if no bats would have died or got lost at all.

In the fourth and seventh column the recaptures are given as fractions of initial numbers.

TABLE 2

Recaptures of males and females of *M. mystacinus*, 1942–1956 (sampling caves combined).

After	Males			Females		
	Initial number	Recaptured	Fractions of recapture	Initial number	Recaptured	Fractions of recapture
1 year	1072	133	0.1241	621	61	0.0982
2 years	984	94	0.0955	597	40	0.0670
3 years	891	65	0.0729	515	43	0.0835
4 years	793	47	0.0593	465	17	0.0366
5 years	657	37	0.0563	396	14	0.0353
6 years	555	17	0.0306	322	8	0.0248
7 years	489	14	0.0286	313	8	0.0256
8 years	489	15	0.0307	313	8	0.0256
9 years	377	9	0.0239	248	1	0.0040
10 years	290	6	0.0207	208	1	0.0048
11 years	200	4	0.0200	135	1	0.0074
12 years	142	1	0.0070	100	0	0.0000
13 years	69	0	0.0000	51	0	0.0000

IV. MATHEMATICAL CONSIDERATIONS[1]

1. PROPERTIES OF THE POPULATION

a. Age–distribution

The population of bats is examined at regular intervals of one year, at a time about half-way between the yearly appearance of young animals. We will devise here a more or less idealized picture of the population at the time of examination which will allow us to define and calculate its most important properties.

At the time of examination the population consists of a number of distinct age-classes, animals belonging to the same class having approximately the same age, while animals belonging to different classes have ages that differ a whole number of years. We will denote the age-class of an animal with the symbol t, starting with $t = 1$ for the youngest animals that have reached an age of about six months at the

[1] The mathematical treatment has been worked out by the third author.

time of examination. Consequently, the age of an animal belonging to class t is approximately $t - \frac{1}{2}$ year.

The structure of the population at the time of examination is described completely by giving the relative frequencies of the various age-classes, for males and females separately. These relative frequencies will be denoted by the symbols m(t) for males and f(t) for females. By summing over all possible values of t we obtain the quantities

$$m = \sum_t m(t) \tag{1}$$

$$\text{and} \quad f = \sum_t f(t) \tag{2}$$

which denote the relative frequencies of males resp. females of all ages. Obviously $m + f = 1$.

b. Mortality of adults

One of the fundamental properties of a population is the mortality rate. This property can be expressed in several ways. We will use for this purpose a quantity p, called the probability of survival, and defined as the probability that an individual, alive at a certain time, will live another year. When this probability is high, the mortality is low, and vice versa.

In general it will not be possible to characterize a population by a single value of p, valid for all its individuals, since this quantity is influenced by a great number of factors. There will be, for instance, differences between animals of different ages, and between males and females. Even animals of the same age and sex will not always have the same chance of living another year. Moreover, owing to changes in environmental conditions, this chance will vary from year to year. It is, however impossible to include all these possibilities in our considerations, as the problem will then become quite unmanageable. We will therefore assume that a single value of p holds for all animals of the same age and the same sex, and that this value remains unchanged throughout the period of investigation. In other words, we only allow for differences between the various age-classes and between the two sexes. The age-class is indicated in the same way as above, the sex is indicated by adding an index m or f to the symbol p. Consequently, $p_m(t)$ denotes the chance of living another year of a male belonging to age-class t, $p_f(t)$ the chance of living another year of a female belonging to age-class t.

At this point we must remind the reader that, owing to our way of sampling, we only observe animals that are at least six months old. To these animals we apply the term adults, and accordingly we may say that our definitions so far only concern the mortality of adults. How-

ever, as will appear later on, it is possible, upon a few additional assumptions, to obtain some knowledge concerning mortality in the first half year.

c. Relation between age-distribution and mortality

To establish a relation between the quantities defined above, we must make some assumptions concerning the nature of the population. Here again we are confronted with a great many possibilities: the population may be growing or declining, there may be a continuous shift in age-distribution or sex ratio, etc. Once more, however, we will simplify the problem by assuming that the population is stationary, i.e. that age-distribution, sex ratio and size of the population do not change in the long run.

To avoid misunderstanding it must be pointed out that this assumption is a statistical hypothesis, and does not exclude stochastic variations in the characteristic quantities in the course of time. The actual values may vary from year to year but these values must be grouped around a mean value which remains constant. Accordingly, the symbols $m(t)$ and $f(t)$ henceforth do not indicate actual values but expected values which will be approximately realized only by pooling observations over a very long period.

If the population is stationary a simple relation between age-distribution and mortality holds. Consider the males of age-class t, having a relative frequency $m(t)$ and a chance of survival $p_m(t)$. After one year the relative frequency of the survivors is expected to be $m(t)p_m(t)$. But as these survivors constitute age-class $t + 1$ of the population after one year, their relative frequency must be equal to $m(t + 1)$, since, according to the assumption, the age-distribution of the population has not changed. Thus we obtain the relation

$$m(t)p_m(t) = m(t + 1) \tag{3}$$

for males, and in the same way the relation

$$f(t)p_f(t) = f(t + 1) \tag{4}$$

for females.

By means of the relations (3) and (4) we can calculate the relative frequencies of each age-class, if those of the first age-class and the probabilities $p_m(t)$ and $p_f(t)$ of each class are known. Applying (3) and (4) repeatedly for successive values of t, we obtain the following results:

$$m(t) = m(1) \prod_{\tau=1}^{t-1} p_m(\tau) \tag{5}$$

$$f(t) = f(1) \prod_{\tau=1}^{t-1} p_f(\tau) \tag{6}$$

Of particular interest is the special case, where the probability of survival is the same for all age-classes, and consequently attains only two values, to be denoted by p_m and p_f. In this case (5) and (6) simplify to

$$m(t) = m(1)\, p_m^{t-1} \tag{7}$$

$$f(t) = f(1)\, p_f^{t-1} \tag{8}$$

By summing over all possible values of t we obtain the quantities m and f defined by (1) and (2):

$$m = \sum_t m(t) = m(1) \sum_t p_m^{t-1} = \frac{m(1)}{1-p_m}$$

$$f = \sum_t f(t) = f(1) \sum_t p_f^{t-1} = \frac{f(1)}{1-p_f}$$

and, substituting these results in (7) and (8), we find:

$$m(t) = m\, p_m^{t-1}\, (1-p_m) \tag{9}$$

$$f(t) = f\, p_f^{t-1}\, (1-p_f) \tag{10}$$

The relations (9) and (10) show that in the special case for which these relations are valid the age-distribution of the whole population is determined completely by three fundamental quantities:

1. the probability of survival of males p_m;
2. the probability of survival of females p_f;
3. the sex ratio m/f.

d. Juvenile mortality

We will now consider the mortality rate during the first six months of life, called juvenile mortality, which, like the mortality of adults, will be expressed by means of a probability of survival, indicated by $p_m(0)$ for juvenile males and by $p_f(0)$ for juvenile females. To get figures comparable with those for adults these probabilites should refer to a period of the same length, i.e. one year. Therefore, the probability of survival during the first half year will be defined as $\sqrt{p_m(0)}$ for males and $\sqrt{p_f(0)}$ for females.

Is m(0) the relative frequency of new-born males and f(0) that of new-born females (both quantities referring to the population at the time of sampling as unity), then the probability of survival during the first half year is given by

$$\sqrt{p_m(0)} = \frac{m(1)}{m(0)} \tag{11}$$

$$\text{and } \sqrt{p_f(0)} = \frac{f(1)}{f(0)} \tag{12}$$

We will restrict ourselves to the case in which the probability of survival for adults is independent of age. Then the quantities m(1) and f(1) can be calculated from (9) and (10):

$$m(1) = (1-p_m)m \tag{13}$$

$$f(1) = (1-p_f)f \tag{14}$$

The quantities m(0) and f(0) depend upon three factors: the number of females alive at the time of birth, the number of young animals produced by each female, and the sex ratio at birth. Assuming a constant mortality rate during the year, the relative frequency of females of age-class t, alive at the time of birth, will be $f(t)\sqrt{p_f}$. The mean number of young animals produced by a female may in general depend upon the age of the female, and will therefore be denoted by the symbol n(t), where t indicates the age-class of the female. Finally, a fraction μ of the total number of new-born animals will be males, a fraction φ will be females. Using this notation, the quantities m(0) and f(0) are given by

$$m(0) = \mu \sum_t n(t) \quad f(t) \quad \sqrt{p_f} \tag{15}$$

$$f(0) = \varphi \sum_t n(t) \quad f(t) \quad \sqrt{p_f} \tag{16}$$

Substituting (13) and (15) in (11), and (14) and (16) in (12) we obtain:

$$\sqrt{p_m(0)} = \frac{(1-p_m)\,m}{\mu \sum_t n(t) f(t) \sqrt{p_f}} \tag{17}$$

$$\sqrt{p_f(0)} = \frac{(1-p_f)f}{\varphi \sum_t n(t) f(t) \sqrt{p_f}} \tag{18}$$

To calculate the probability of survival during the first half year from these formulae we must make assumptions concerning the unknown quantities μ, φ and n(t).

There are two equally likely assumptions that lead to the elimination of μ and φ. In the first place we can assume that the factors causing a difference in mortality between the two sexes are acting only in adult life and not during the first half year. In that case $p_m(0)$ and $p_f(0)$ become equal, say = p(0), and (17) and (18) can be combined to

$$\sqrt{p(0)} = \frac{(1-p_m)m + (1-p_f)f}{\sum_t n(t)\, f(t) \sqrt{p_f}} \tag{19}$$

In the second place we can assume that at birth there is a sex ratio = 1 in which case $\mu = \varphi = \frac{1}{2}$. By adding (17) and (18) and putting

$$\frac{\sqrt{p_m(0)} + \sqrt{p_f(0)}}{2} = \sqrt{p(0)}$$

we obtain once more relation (19), the quantity $\sqrt{p(0)}$, however, signifying in this case the mean value of $\sqrt{p_m(0)}$ and $\sqrt{p_f(0)}$.

As regards the birth-rate it is known (see elsewhere in this paper) that the majority of female bats produce one young each year, except in their first year when at least part of them do not become pregnant. We will assume therefore $n(t) = 1$ for all values of $t > 1$, while as regards $n(1)$ we will consider the two extreme possibilities $n(1) = 1$ and $n(1) = 0$.

In the first case

$$\sum_t n(t)\ f(t) = f, \qquad \text{consequently}$$

$$\sqrt{p(0)} = \frac{(1-p_m)m + (1-p_f)f}{f\sqrt{p_f}} \tag{20}$$

In the second case

$$\sum_t n(t)\ f(t) = f - f(1) = f - (1-p_f)f = fp_f,$$

consequently

$$\sqrt{p(0)} = \frac{(1-p_m)m + (1-p_f)f}{fp_f\sqrt{p_f}} \tag{21}$$

Relation (20) gives a lower boundary, relation (21) an upper boundary for $\sqrt{p(0)}$.

e. Expectation of life for adults

If we assume once more that the probability of survival for adults is independent of age, their expectation of life (i.e. the mean number of years they may expect to live) within each sex has a constant value too. To calculate this value, however, this assumption is not sufficient, as it tells nothing about the behaviour of the mortality rate in the course of a year. An additional assumption concerning this behaviour is needed, and once again we choose the simplest possibility, i.e. that the mortality rate remains constant throughout the year.

Now let t signify the length of time in years that has passed since a given moment (here t no longer assumes only integral values as before, but may have any value $\geqslant 0$), and let y be the fraction of the population of adults at time $t = 0$ that is expected to survive at time t, then the expectation of life is given by

$$E(t) = \int_0^\infty y\,dt \tag{22}$$

If the mortality rate is constant, the number of animals dying in the interval from t till $t + dt$ will be proportional to the number of animals alive at the beginning of the interval and to the length of the interval.

Therefore

$$- dy = \text{Const. } y\, dt \tag{23}$$

Integrating (23) between the limits $t = 0$, $y = 1$ and $t = 1$, $y = p$ (where $p = p_m$ for males and $p = p_f$ for females), we obtain:

$$y = p^t \tag{24}$$

Substitution of (24) in (22) and evaluation of the integral gives

$$E(t) = -\frac{1}{\ln p} \tag{25}$$

It must be pointed out that (25) gives the length of time in years that an adult animal can expect to live from a given moment onwards. To obtain the mean age of adult animals we must add the time it takes to attain the adult stage, in our case 0.5 year.

2. ESTIMATION OF POPULATION PARAMETERS FROM RECAPTURE DATA

a. Estimation of the probability of survival

As explained in the first part of this paper the sampling method finally results in a series of fractions of recapture after an increasing number of years (cf. page 68). To use these data for the estimation of population parameters, we will assume that the probability of survival and the rate of capture are independent of age and remain unchanged throughout the period of investigation. As it is possible to treat the data for males and females separately, we need make no restrictions regarding the influence of sex.

If p is the probability of survival (for males $p = p_m$, for females $p = p_f$), and q the probability of being caught (for males $q = q_m$, for females $q = q_f$), the probability of recapturing an animal t years after it has been ringed is $p^t q$, and the fraction of recapture after t years, denoted by $r(t)$, will be an unbiased estimator of this probability:

$$\mathscr{E} r(t) = p^t q \tag{26}$$

If the investigation is continued during n years, the problem is: to obtain an estimate of p and q from the n values $r(1) \ldots r(n)$.

Several methods to solve this problem are available, but they all have the drawback that the computational labour rapidly becomes enormous as the number of years increases. Moreover, most of them have been developed for conditions that are not fulfilled in our case. Therefore we have turned to a method which, though it may lead to slightly erroneous results, has the advantage that the necessary calculations are very easy for any number of years.

This method is suggested by the form of equation (26). Taking the

logarithm, the righthand side of (26) becomes a linear function of t:

$$\log \mathscr{E} r(t) = t \log p + \log q \tag{27}$$

This suggests the following procedure: plotting log r(t) against t, fitting a straight regression line to the n available points, and finally calculating p from the regression coefficient and q from the intersection with the vertical axis.

It is clear that the accuracy of the method depends upon the number of points included in the calculations. The chance of recapturing an animal after t years, and accordingly the expected number of recaptures after t years decreases with increasing values of t. Consequently for large values of t the data will have large relative errors. To restrict the influence of this effect we include in our calculations only those values of t for which the expected number of recaptures is not smaller than 10.

b. Verification of the method

There are several reasons why the procedure described above is not quite correct. Owing to the complicated nature of the calculations involved, it is impossible to evaluate theoretically the errors introduced by certain approximations underlying the method. For the same reason it is impossible to calculate the variances of the estimates of p and q. To obtain nevertheless some information concerning the accuracy of the method, a sampling experiment was carried out with an artificial population with known properties. The results were treated in the way described above, and the estimates of p and q could be checked against their real values.

The sampling experiment may be described here in a few words. Each experiment was started with a set of 1000 punchcards, representing the bat population, of which 600 were characterized as males, 400 as females. Each card carried a number of random digits punched in different columns (fig. 2) so that random samples from the set could be taken. The events occurring in the population in the course of a year were represented by the following manipulations:

1. Cards were selected at random with a probability of 0.2, and marked as "caught".
2. Cards were selected at random with a probability of 0.2 for males and 0.3 for females, marked as "dead", and eliminated from the population.
3. New cards were added, representing new-born animals. Their number was determined by means of another stochastic process, defined in such a way that the population would be stationary.

The whole sequence of events, repeated ten times to represent an investigational period of ten years, constituted a single experiment, and in

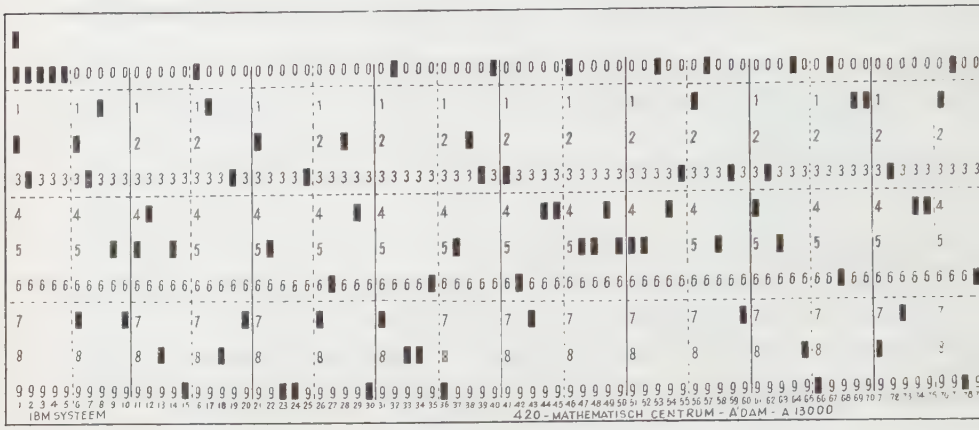

Fig. 2. I.B.M. punchcard as used for sampling experiments (cf. page 75).

this way 18 experiments in all were carried out. The results are shown in Table 3. They prove that at least under the conditions of the sampling experiment the theoretical fallacies of the method introduce no serious error, and that the estimates of p and q are reasonably accurate.

c. Verification of assumptions

To derive the results in the preceding paragraphs a number of assumptions have been made, and we will now consider whether these assumptions can be verified, and if so, to what extent.

In the first place it has been assumed that the population is stationary, i.e. that is does not change in the long run with respect to size, age-distribution and sex ratio. Constancy of the age-distribution cannot be tested as this property is not directly observed in the samples. The two other properties, however, allow statistical tests, based upon the size and the sex ratio of the sample. Constancy of the size can be tested by means of the rank correlation statistic S (KENDALL 1948,) constancy of the sex ratio by means of the statistic χ^2. In both tests the final criterion is the probability P of exceeding in a series of samples from a constant population the actual value of S or χ^2 respectively. As long as this probability exceeds the value 0.05, constancy of size or sex ratio respectively may be accepted.

Secondly, it has been assumed that the probability of survival and the rate of capture are independent of the animals' age and of the year of sampling. If this assumption is valid, the points in the log r(t)/t diagram should show no apreciable systematic deviation from the straight regression line. If this is actually found to be the case, it may be said that the assumption at least is not disproved. There may be other

TABLE 3

Estimates of p and q in the sampling experiment, calculated by means of the regression line method.

Exp. nr.	*Males*		*Females*	
	p	*q*	*p*	*q*
1	0.777	0.206	0.727	0.169
2	0.817	0.194	0.604	0.241
3	0.824	0.206	0.716	0.221
4	0.821	0.175	0.649	0.239
5	0.813	0.184	0.718	0.183
6	0.834	0.168	0.701	0.221
7	0.816	0.189	0.709	0.190
8	0.776	0.214	0.658	0.234
9	0.802	0.184	0.712	0.213
10	0.842	0.172	0.677	0.192
11	0.792	0.210	0.747	0.186
12	0.823	0.172	0.688	0.194
13	0.798	0.185	0.809	0.135
14	0.781	0.208	0.638	0.261
15	0.775	0.219	0.650	0.244
16	0.761	0.222	0.732	0.221
17	0.776	0.209	0.623	0.235
18	0.778	0.196	0.722	0.189
Mean	0.800	0.195	0.693	0.209
Standard deviation	0.024	0.017	0.050	0.032
Real value	0.800	0.200	0.700	0.200

conditions resulting in a regression line that, within the limits of experimental error, is not distinguishable from a straight line. But as long as no evidence of the contrary has been obtained, constancy of p and q may be accepted as the simplest hypothesis in agreement with the experimental results.

In the third place, assumptions have been made concerning the mortality rate during the year and the birth rate. Neither of these assumptions is verifiable, as observations are only made at yearly intervals and no observations at the time of birth are available. However, any mistake in these assumptions will affect only our estimates of the juvenile mortality and the expectation of life.

Finally, attention may be drawn here to a few points that have not been mentioned so far. It is an inevitable consequence of the capture-recapture technique that the data obtained refer to marked animals only. Therefore the properties derived from these data are strictly

speaking properties of a population of marked animals that might differ from the investigated population in certain aspects. In our case, for instance, it is conceivable that the procedure of banding influences the death rate. Without additional data from other sources, however, it is impossible to obtain information upon this point. Another point which, with our present technique, will have to remain undecided, is the interpretation of the term mortality. Our observations show only a loss of animals, the largest part of which is undoubtedly due to death. Emigration, however, may also be responsible for part of this loss, and here again the capture-recapture technique fails to provide sufficient information.

V. RESULTS AND CONCLUSIONS

1. CONSTANCY OF THE POPULATION

According to the indications in the preceding chapter (page 76), constancy of the size and the sex ratio in the population has been tested by means of the rank correlation test of KENDALL and the χ^2-test respectively. For the period 1942–1956 the first yields a value of $P = 0.582$, the second a value of P between 0.05 and 0.10. From these values it may be concluded that the size as well as the sex ratio in the population have not changed significantly during this period.

Constancy in age distribution cannot be tested, the age of the individuals being unknown, but it is unlikely that the age distribution should have changed, while no changes in size and sex ratio could be established.

2. ACCURACY OF THE REGRESSION LINES

In the figs. 3, 4 and 5 the logarithm of the fraction of recapture multiplied by 100 is plotted against the number of years after ringing. The fractions are taken from Table 2 (page 68), but only those have been used for which the initial number is sufficiently high that at least 10 recaptures may be expected (cf. page 75).

The graphs show that the regression lines represent the observed recapture fractions rather satisfactorily.

3. SURVIVAL RATE AND CORRESPONDING POPULATION PROPERTIES

a. Survival rate and rate of capture

As figs 3–5 show, the individual points do not deviate systematically

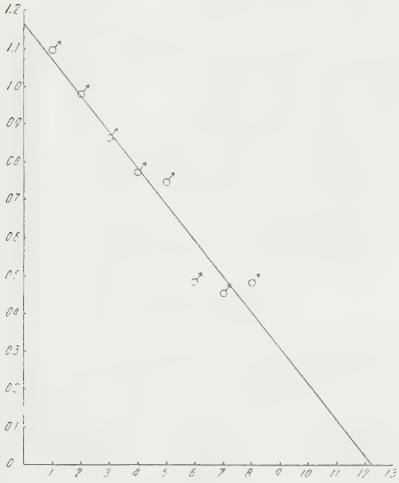

Fig. 3

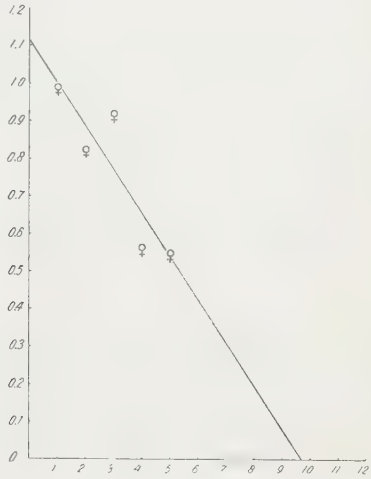

Fig. 4

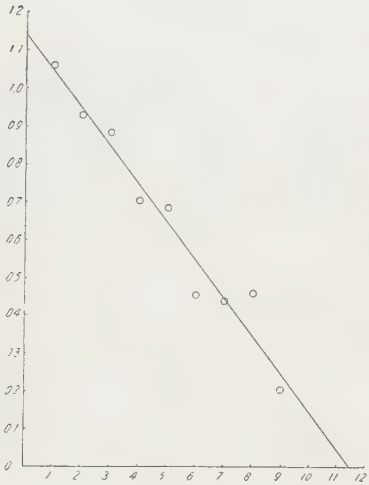

Fig. 5

Fig. 3–5. Logarithm of recapture fractions multiplied by 100 (vertical axis) plotted against years after ringing (horizontal axis); regression lines fitted to the points (cf. page 75). Fig. 3: males; fig. 4: females; fig. 5: sexes combined

from a straight regression line. As is pointed out on page 76, the simplest explanation of this fact is constancy of the survival rate and the rate of capture. Consequently, we feel justified in representing animals of different age-classes by single values of p (= the probability of survival, defined on page 69) and q (= the probability of being caught, cf. page 74), valid for the whole period of investigation. As is shown on page 75 estimates of these quantities can be obtained from the slope of the regression line and its intersection with the vertical axis. They are recorded in Table 4, n denoting the number of points on which the regression line is based.

TABLE 4

Rates of survival (p) and capture (q) in *M. mystacinus*, 1942–1956 (sampling caves combined).

	Males	*Females*	*Sexes combined*
n	8	5	9
p	0.803	0.767	0.795
q	0.146	0.131	0.140

The sexes show slightly different values of p and q. However, these differences are not significant, if tested by means of STUDENT's t-test, using the standard deviations of p and q as recorded in Table 3 (page 77). Therefore, we may pool the data of the sexes and compute a joint regression line for the population as a whole, which is consequently based on larger and therefore more accurate numbers. Owing to this combination we can use 9 points for this line, resulting in estimates of 0.795 for the survival rate and 0.140 for the of rate capture.

By means of this survival rate, the expected numbers of survivors out of an initial number of 1000 full-grown animals ringed have been calculated and recorded in Table 5. The survival rate probably being constant, the numbers of survivors recorded in Table 5 would have been the same, if we had started with 1000 full-grown specimens, all belonging to the first yearclass.

TABLE 5

Years after ringing	*Survivors out of 1000 ringed*	*Years after ringing*	*Survivors out of 1000 ringed*
0	1000	11	79
1	795	12	63
2	632	13	50
3	502	14	40
4	399	15	32
5	317	16	25
6	252	17	20
7	200	18	16
8	159	19	13
9	126	20	10
10	100	21	8

b. Life span

If we choose as a definition of *life span:* the number of years after which only 10 individuals out of 1000 ringed are expected to be still alive, the value of this quantity found by extrapolation is 20 years (Table 5).

In extrapolating it is assumed that survival rate remains constant throughout life, even in the oldest individuals. We are not yet able to decide definitely, whether old age affects survival rate in bats or not. The only observable information on life span originates from the maximal numbers of years (within the limit of the period of investigation, i.e. 13 years) during which recaptured animals have been wearing their ring. These are, as yet, for *M. mystacinus* males 13 years and females 11 years. The real age of the animals at the date of recapture is not known, because their age at the date of ringing could not be determined.

c. Expectation of life

Substituting the value p = 0.795 (page 80) in formula (25) on page 74, we obtain for the *expectation of life* the value 4.4 years. Accordingly the *mean length of life* of adults is 4.9 years (cf. page 74).

4. SEX RATIO AND AGE-DISTRIBUTION

As the rate of capture is found to be not significantly different in the sexes (page 80), the *sex ratio* in the population during the period of investigation can be estimated by counting males and females in the samples. Out of 2027 full-grown specimens, 1288 were males. Consequently the sex ratio is $\frac{1288}{739} = 1.74$ and the fraction males $\frac{1288}{2027} = 0.635$.

As the survival rates are equal in adult males and females (cf. page 80), the sex ratio in each yearclass will be equal to this ratio in the total population.

Survival rate and sex ratio being known, the age-distribution of the population can be calculated using the formulae (9) and (10) on page 71. It is recorded in Table 6.

TABLE 6

Age-distribution in winter population of *M. mystacinus* (sampling caves combined).

Age in years	*m(t) fraction* ♂♂	*f(t) fraction* ♀♀	*m(t) + f(t)*	*Age in years*	*m(t) fraction* ♂♂	*f(t) fraction* ♀♀	*m(t) + f(t)*
½	0.130	0.075	0.205	7½	0.026	0.015	0.041
1½	0.103	0.059	0.162	8½	0.021	0.012	0.033
2½	0.082	0.047	0.129	9½	0.016	0.009	0.025
3½	0.065	0.038	0.103	10½	0.013	0.007	0.020
4½	0.052	0.030	0.082	11½	0.010	0.006	0.016
5½	0.041	0.024	0.065	12½	0.008	0.005	0.013
6½	0.033	0.019	0.052	13½	0.007	0.004	0.011

5. JUVENILE MORTALITY

In the Netherlands bats are born in June or July. Thus the youngest individuals in the samples are 6 months old. Consequently, the values of survival rate and of the other population properties, dealt with sofar, relate to individuals aged 6 months and more, i.e. to full-grown animals only.

Juvenile mortality (the mortality rate between birth and the following January) in a stationary population is calculable from the annual survival rate of adults and their known fertility (cf. page 71). Bats of the species involved here produce one young per annum. According to histological investigation of the reproductive organs during hibernation, the females are likely to become pregnant for the first time at the end of their second year (SLUITER, 1954). If this is true, formula (20) on page 73 applies and yields a value of 0.79 for juvenile survival, i.e. 0.21 for juvenile mortality.

If, on the other hand, we would assume that all the females belonging to the first yearclass already have a young, juvenile mortality could be calculated by means of formula (21) (page 73) as 0.37.

The histological evidence mentioned above does not exclude the possibility that a small number of females which are born in the very beginning of the breeding season should reach sexual maturity before the next spring. If the truth about the reproductive rate were between the limits mentioned above, meaning that an unknown (but small) percentage of the first year females becomes pregnant, the juvenile mortality rate would probably be a little higher than 0.20, but certainly lower than 0.40.

On the assumption that the survival rate remains constant in the course of a year, the adult survival during six months is $\sqrt{p} = \sqrt{0.795} = 0.891$. This corresponds with a mortality rate of 0.11 per 6 months. Therefore, juvenile mortality is found to be at least twice as high as adult mortality over the same period.

6. RATE OF CAPTURE

The probability of an animal being caught, referred to here as the "rate of capture" is, of course, lower than 1, as many bats are likely to be overlooked during our routine visits (cf. page 66). Pooling the data for all sampling caves and all years of investigation, we found for the rate of capture for males and females combined the value 0.140 (cf. Table 4).

We will now consider two factors – apart from the activity of the collectors – on which the rate of capture depends:

1. The opportunities which a cave provides for a bat to find a hiding place.
2. The behaviour of the bats in selecting a site to sleep.

Ad 1. The sampling caves have certainly different values as hiding places for bats. While the numbers of captures in a single cave are generally too low to allow estimation of the rate of capture in each cave separately, only a comparison between Slavante as our largest sampling cave and the other caves combined is possible. The basic data for this comparison are recorded in Tables 7 and 8, the results in Table 9.

TABLE 7

Recaptures of *M. mystacinus* in Slavante, 1942–1956

After	*Males*			*Females*		
	Initial number	*Recaptured*	*Fraction recaptured*	*Initial number*	*Recaptured*	*Fraction recaptured*
1 year	352	69	0.1960	271	37	0.1365
2 years	344	44	0.1279	269	28	0.1041
3 years	306	24	0.0784	240	30	0.1250
4 years	241	15	0.0622	202	14	0.0693
5 years	223	13	0.0583	185	9	0.0486
6 years	190	9	0.0474	—	—	—

TABLE 8

Recaptures of *M. mystacinus* in the other sampling caves combined, 1942–1956

After	*Males*			*Females*		
	Initial number	*Recaptured*	*Fraction recaptured*	*Initial number*	*Recaptured*	*Fraction recaptured*
1 year	720	64	0.0889	350	24	0.0686
2 years	640	50	0.0781	328	12	0.0366
3 years	585	40	0.0666	275	14	0.0509
4 years	552	32	0.0580	263	3	0.0114
5 years	434	24	0.0553	—	—	—
6 years	365	8	0.0219	—	—	—

TABLE 9

Rates of survival (p) and of capture (q) of *M. mystacinus*, calculated from the data of the Tables 7 and 8; n relates to the number of points on which regression lines are based.

	Slavante			*Other sampling caves combined*		
	Males	*Females*	*Sexes combined*	*Males*	*Females*	*Sexes combined*
n	6	5	7	6	4	8
p	0.758	0.781	0.795	0.792	0.603	0.789
q	0.220	0.189	0.190	0.126	0.123	0.109

In these tables the data for males and females have been treated separately, but since here again no significant differences can be detected (the abnormally low value of p for females in the "other caves combined" is due to the small value of n), we need only consider the values of p and q for the combined sexes.

There is a remarkable agreement between the values of the survival rate, an additional argument in favour of the accuracy of the method, as a difference in survival could not be expected. On the contrary, there is a large (and statistically significant) difference between the values of the rate of capture, this quantity being in Slavante nearly twice as high as in the other caves combined. This phenomenon can easily be explained, as the walls and ceilings of the corridors in Slavante are extraordinarily smooth, and crevices are rare. For this reason the bats are more easily seen there than in the majority of the other sampling caves.

Ad 2. As is shown on page 80 the bats have a rate of capture of 0.14, meaning that at each visit on the average 86 % of the then living individuals have not been captured. This percentage being so high may be explained in several ways connected with the behaviour of the bats. First, they may have slept in a sampling cave, but have been overlooked because they were hiding in a crevice or (and) sleeping in a corridor which has not been examined by the collectors. The latter may happen easily in very large caves like Slavante. Secondly, the bats may have slept somewhere outside a sampling cave (probably in another cave of the same area), at the time of examination (cf. page 66.).

We are not able to decide which of these two possibilities has occurred most frequently. According to the experiments of De Wilde and Van Nieuwenhoven (1954), the majority of the bats hibernating in a certain cave can only be found after a third or fourth close examination of the place. Therefore, we are inclined to believe that many of the individuals we failed to find at the annual routine visits to the sampling caves, have still been hiding there.

7. POPULATION SIZE, DISTRIBUTION AREA, SEASONAL MIGRATION

By multiplying mean sample sizes with 1/q mean *sizes of populations* during the period of investigation may be estimated, as recorded in Table 10.

Apparently, in *M. mystacinus* the size of the population of the combined sampling caves has remained constant from 1942 till now at a level of about 1200 individuals.

The sampling caves contain an unknown fraction of the total population, hibernating in the cave area of Southern Limburg. It may be

TABLE 10

Estimates of mean annual population size, 1942–1956

	Mean sample size	*Rate of capture (q)*	*Mean population size*
Slavante	66	0.190	375
Other sampling caves combined	96	0.109	880
Totals	162	—	1255

concluded that the vast cave labyrinths in this area of about 50 square miles (cf. page 64) belong to the most important hibernating quarters of *M. mystacinus* in Europe (cf. Bels, 1952).

Owing to the banding of a great number of individuals, something more may be said concerning *the distribution area* and the *seasonal migrations* of this population. Between May and October practically no bats are found in the caves. From the topography of s.c. "foreign returns" (recoveries from places outside the cave area) recorded by Bels (1952) and extended by us, it is inferred that during springtime the population is spreading mostly in Northern direction over an area of about 300 square miles (fig. 6). No further details of the summer haunts are known as yet.

It is supposed that the greater part of the population returns to the cave area to hibernate, but no quantitative data are available to sustain this hypothesis. Still less can be proved quantitatively concerning the assumption that the greater part of the individuals should return annually to the cave where they have hibernated before. There are actually a number of cases in which a bat was recaptured during several subsequent years in the cave where it had been banded, but it is very difficult to investigate this phenomenon, since the rate of capture in the majority of the caves has been as low as about 0.14.

According to De Wilde and Van Nieuwenhoven (1954) bats wake up spontaneously several times during the hibernation period. It is probable that some of them disappear into crevices to continue their hibernation, but that other individuals emerge from their hiding places to go to sleep again on a site in the same cave where they can easily be seen. These habits may cause the false impression that the population of a cave is shifting during one winter (cf. Bels 1952).

Fig. 6. Foreign returns of *M. mystacinus* (black dots) banded in the cave area (⊕), 1942–1956. After Bels (1952), with 8 additional data from the period 1952–1956.

VI. SUMMARY

Annual mortality rates and other population properties are estimated in a stationary hibernating population of *Myotis mystacinus* in the cave area of Southern Limburg (fig. 1).

The basic data for this study originate from recaptures of ringed bats in 18 sampling caves during a period of 13 years (cf. Tables 1 and 2). Sample sizes vary, but do not show a tendency to decrease or increase steadily. They are grouped around a mean value of 162 individuals. The sex ratio is fairly constant at 1.74, the males being the more numerous.

By plotting the logarithm of fractions of recapture against time after ringing regression lines are computed (figs 3–5), and from these lines survival rates and rates of capture are estimated. The accuracy of the method is found fairly acceptable when checked by marking-recapture experiments with a model of a bat population made of I.B.M. punch cards (cf. Table 3, fig. 2).

Annual mortality rates are found to be equal in the sexes. They are constant at a level of about 0.20 during adult life (Table 4). In juveniles from birth till the age of 6 months this rate is nearly twice as high as that in adults during the same length of time. An estimate of life span is calculated as 20 years in this species (Table 5). Mean longevity is in the order of magnitude of 5 years. Age-distribution is recorded in Table 6.

Estimation of *the rate of capture* during the annual routine inspections of the sampling caves gives a mean value as low as 0.14, partly originating from the habit of many individuals to hide in crevices when hibernating. A difference between the rates of capture in different caves has been established (cf. Table 9) and could be explained by ecological characters of these caves.

Mean annual *population size* in the combined sampling caves from 1942–1956 is calculated as about 1200 individuals (cf. Table 10).

Finally some additional data on *distribution area* and *seasonal migrations* of the population of the cave area are given (cf. fig. 6).

VII. ACKNOWLEDGEMENTS

In the first place we wish to thank Dr L. Bels (Singapore) for providing us with the main part of the basic data for this study. We owe much to Ir D. C. van Schaik (Maestricht) for guiding us round the caves so many times. We are grateful to Prof. Dr Chr. P. Raven (Utrecht) for reading the manuscript, to Dr D. Chitty and Dr P. H. Leslie (Oxford) for the interest shown in our work. We are indebted to Prof. Dr J. Hemelrijk and Mrs G. Klerk-Grobben of the mathematical centre (Amsterdam) for their advice on the statistical treatment, and to Mr J. Doornbos and Miss H. D. Sandberg for carrying out the punchcard-experiments at the same Institute. In 1953 and 1954 sampling has been done with a grant of the Netherlands Organisation for pure research (Z.W.O.).

VIII. REFERENCES

Bels, L., 1952: Fifteen years of bat banding in the Netherlands. Thesis, Utrecht. Also: Publicaties van het Natuurhistorisch Genootschap in Limburg, Reeks V.

HEERDT, P. F. VAN and J. W. SLUITER, 1953: The results of bat banding in the Netherlands in 1952 and 1953. Natuurhist. Maandblad, **42**.

HEERDT, P. F. VAN and J. W. SLUITER, 1954: idem in 1954. Ibidem **43**.

HEERDT, P. F. VAN and J. W. SLUITER, 1956: idem in 1955. Ibidem **45**.

KENDALL, M. G., 1948: Rank Correlation Methods (Griffin & Co., London).

SLUITER, J. W., 1954: Sexual maturity in bats of the genus *Myotis*. II Females of *M. mystacinus*..... Proc. Kon. Ned. Akad. Wet. Amsterdam C, **57**.

WILDE, J. DE and P. J. VAN NIEUWENHOVEN, 1954: Waarnemingen betreffende de winterslaap van vleermuizen. Publicaties van het Natuurhistorisch Genootschap in Limburg, Reeks VII.

ANNUAL REPORT OF THE ZOOLOGICAL STATION OF THE NETHERLANDS ZOOLOGICAL SOCIETY FOR THE YEAR 1955

The research programme carried out at Den Helder in 1955 again featured the mussel (*Mytilus edulis*) as one of the main objects of investigation. The work on this species is gradually gaining depth. Apart from this, other important work was done, not in the last place by the two research biologists working for the Government Organization for Pure Research.

As to the mussel, a contribution to field work was made by Mr. S. PARMA, Amsterdam, detached by the Government Institute of Fisheries Research for a four months' stay at Den Helder. His task was to find out to what extent the quantity of young mussels in the Wadden Sea could be enhanced by certain measures. The method employed in mussel-farming in this country is that young mussels, so-called seed (mainly mussels in their first year of life), are caught on the natural musselbanks and scattered in certain localities where they have ample room to develop. In due course they are gathered for commercial distribution. Because this is a remunerative trade, musselseed is in great demand and it would be advantageous to find a method by which to increase the available quantities. Theoretically, this might be done in two ways: 1) by increasing the material for primary settlement, so that more spat would have a chance of settling on metamorphosis; 2) by providing a good substratum in the shape of a suitable solid bottom for young mussels that have passed through the period of primary settlement.

For this purpose the Government Institute of Fisheries Research had nets fastened to solid wooden stakes in a few places in the Wadden Sea. In these nets branches of broom (*Sarothamnus*) and of various kinds of trees and strawrope were deposited. The nets were mainly hung in a vertical position at various depths, a small number were placed horizontally. Mr. PARMA was to inspect the spatfall on this artificial substratum, determine the different degrees of density on the various

types of material, and study the growth of the spat in order to form an idea of the length of time during which the mussels remain on this material, or make other observations that might appear to be of interest in connection with the problem involved.

In our previous Annual Report it was already indicated how laborious this investigation is because such a large part of the work involves spat from 250 μ upward, which are invisible to the naked eye. As also the work on mussels up to about 2000 μ continually calls for *measuring*, so much time is taken up by this purely mechanical part that in point of fact an investigation of this kind should not be carried out by a single person. Nevertheless, Mr. PARMA obtained a number of valuable results, also from a purely scientific point of view:

1. The spatfall in the Wadden Sea started rather late in the season, viz., in the second half of June. No spatfall was observed in the autumn, as is sometimes the case in other years.

2. For the settling of the musselspat a certain amount of fouling is of great importance as a secondary substratum. A firbranch will become densely overgrown especially with *Tubularia*, hence it will attract many small mussels; on straw there will be many *Balanus* and consequently small mussels will be attracted. Species which play an important part in this respect are the Hydroids *Laomedea* sp. and *Tubularia larynx*, the barnacles (*Balanus crenatus*), and the red alga *Ceramium rubrum*. Later mussels, settling among the earlier arrivals, have no need of the original secondary substratum, so that the latter gradually becomes less important. *Lanice conchilega*, the sandmason, was favourable to settlement if the species was not present in large numbers; if it was abundant, as was the case on the horizontal net, it had an adverse effect on the eventual numbers of mussels.

3. The production of spat was greatest on the nets farthest from the bottom. Further, the horizontal net, as compared with the vertical ones, produced hardly any spat.

4. Branches and strawrope, driven into the bottom on a tidal flat covered with shallow water, yielded a reasonable production in the slightly deeper parts. Also a herringnet stretched along the bottom was covered moderately well. The growth of the mussels was less here than on the nets in somewhat deeper water along the creeks. The full data on the growth of the mussels are not yet available, however.

Also laboratory work on the mussel was continued.

Mr. HERREBOUT, Utrecht, studied the combined influence of temperature and salinity on the mussel. Before the war much work was done at Den Helder on the combined influence of these factors on the shrimp, *Crangon crangon*, and the shore crab, *Carcinus maenas*. Longevity was then taken as the starting point and it was found that optimal conditions are

obtained if salinity decreases with an increase of temperature and vice versa. Investigations elsewhere have shown that the same holds for other Crustacea which move from the shore to deeper water in winter. Migration in these animals so to speak takes the combined influence of salinity and temperature as a starting point. Naturally, the question arises whether in a non-migratory species a change of temperature is *not* attended with a shift in optimal salinity. Mr. HERREBOUT chose the mussel to work with, and the quantity of water pumped by the animal was taken as a measure to determine the combined influence of temperature and salinity. The quantity of water pumped was found indirectly through determining the decrease in concentration of a suspension of uniform particles of such a size that the mussel could not pass them out. From the work of Dr. TAMMES and Mr. DRAL we know that *Lycopodium* spores can serve as such. Mr. HERREBOUT's investigation presented all kinds of difficulties. The main one was that it is so hard to keep the rate of settlement of the particles constant in the individual experiments. Mr. HERREBOUT obtained indications (but no more) that the salinity at which the rate of pumping attains maximum values is higher at a higher than at a lower temperature, which would indeed indicate a shift of optimal salinity attending a change in temperature, but this shift would be the reverse of what we find in Crustacea. It should be added that the results are not yet to be considered definitely established.

Other work on the mussel was done by Mr. DRAL in consultation with Mr. VERWEY. This investigation centred round the question how the mussel succeeds in retaining or passing out at will the suspended particles of a suitable size which form its food. TAMMES and DRAL (Annual Report for 1952 and their paper in Archives Néerl. de Zoologie, 1955) found that material of a particle size of about 40 μ is retained almost completely, while particles below 5 μ are the more difficult to retain as they become smaller. Particles of sizes between these extremes can apparently be retained and passed out at will. Dr. TAMMES explained these facts by assuming that larger particles stand a better chance of being caught by the stiff laterofrontal cilia than smaller ones, and his view was that the particles adhere to the cilia, as had also been observed by WALLENGREN in the early part of this century. Further, Dr. TAMMES thought that the mussels could make the cilia sticky at will and thus retain or pass out particulate material. The large size of the particles passed out, however, made it appear probable that variation in stickiness alone was not sufficient and that the *position* of the laterofrontal cilia has something to do with the passing out of particles. Mr. DRAL's researches aimed at obtaining data on this point. Through his efforts the movement of the cilia has now been subjected to detailed enquiry, and it is practically certain that their position can indeed be

changed and that it is of vital importance for the retention or passing out of suspended particles.

In connection with the research on mussels we should also mention a visit paid us by Fräulein Brunhild STURCKOW, a former pupil of Prof. ANKEL, Giessen. For some years she has been engaged upon an investigation of the way the mussel attaches itself to its substratum by means of its byssus, and this research has led her to the question of growth and wear of byssus, and of the causes of the differences in thickness of the byssus in individual mussels and their effect on attachment. The object of her visit was to test her findings on animals from different habitats, and at the same time to see something of the work done on mussels at Den Helder.

An entirely different kind of investigation on mussels was made by Miss KOOPMAN, Utrecht, in continuation of preliminary research started in 1953. It was made at the suggestion of Dr. KORRINGA. When a mussel closes its shell, heart pulsation virtually ceases. Miss KOOPMAN tried to find out what precisely causes this reaction. She worked with mussels of about 600–1000 μ, in which the heart can be seen pulsating through the transparant shell. It was found that a decrease in oxygen of the water does not bring about a diminution of pulsation until the oxygen concentration in the surrounding water has become as low as about 20 %. In these circumstances the shell may remain open. The animal's reaction to carbon dioxide is quite different from that on oxygen. Even when the CO_2-concentration in the seawater increases slightly, the rate of pulsation decreases a little and it will fall further according as the carbon dioxide concentration increases, also if the water remains saturated with oxygen. These results do not constitute definite *proof* that carbon dioxide actually provides the stimulus that stops pulsation in mussels which have their shells closed (we know nothing about the carbon dioxide tension in the blood), but they do indeed make this quite probable. In a recent study, which did not, however, involve *changing* quantities of carbon dioxide, SCHLIEPER arrived at the same conclusion.

Miss GEHRELS, Amsterdam, had hoped to find out particulars of the life history of the small cuttlefish, *Sepiola atlantica*. Unfortunately, she did not succeed in keeping her specimens in good condition. After some weeks she therefore transferred her attention to another subject: the factors which induce the appearance of a particular colour pattern in the common cuttle, *Sepia officinalis*. The colour patterns of *Sepia*, which have been described by HOLMES, are obviously a response to changes in the animal's environment. Miss GEHRELS gave her special attention to one particular pattern characterized by a white spot approximately in the centre of its back. This spot appears when the animal lies quietly

on the bottom, especially if light-coloured objects like shells and pebbles cover the bottom. Miss GEHRELS found that it tends to appear even more often as a response to shells painted yellow rather than white, but hardly, if at all, as a reaction to dark-coloured ones. A striking point was that not only did the animals produce the white spot in response to light-coloured objects, but they also showed a preference for taking up positions near these objects. It rather looked as if in this way they sought to promote their own camouflage. Further experiments seemed to indicate, however, that they were also partial to positions near dark-coloured shells, where consequently they would not produce their white spot. To sum up the results it would appear that *Sepia* shows a preference for a highly structurated bottom, and in addition produces its white spot if on this bottom there is a white (or yellow) coloured object. This investigation to some extent followed up the work done by KÜHN, though KÜHN used the colour adaptation of *Sepia* to the bottom as a means to study the animal's sensitivity to colours. Miss GEHRELS also tried to determine the influence of the size and number of white objects, but her researches did not yet produce an answer to this and other questions. The work takes up an enormous amount of time as the various specimens differ in the degree to which they tend to exhibit particular colour patterns. It would certainly be worth while, however, to continue this item in the near future.

Mr. JANSSEN, Utrecht, studied the influence of temperature on geotaxis in the flat periwinkle, *Littorina obtusata*. We know from the work of BARKMAN and EBBINGE WUBBEN, the results of which were published in one of the recent issues of Archives Néerlandaises de Zoologie, that these animals are kept within the tidal zone by a strong negative geotaxis, which causes them to seek higher levels in the water, and a negative phototaxis and their dependence on Fucaceae, which keep them below the surface. One may expect, however, that this strong negative geotaxis is eliminated in winter, so that the animals are enabled to seek lower levels. The investigation was made to establish this point. In the course of it evidence was obtained that the negative sign of the geotaxis is not changed until the temperature falls to little above zero. As soon as the temperature approaches zero and particularly at 0° and below, the animal's geotaxis becomes positive. In a manner of speaking, the animals are detained as long as possible within the tidal zone, which offers good conditions as regards food, and they do not leave it until frost sets in. We hope this study will be published in the Archives.

Mr. HAECK, Utrecht, endeavoured to bring his investigation mentioned in our previous Annual Report, on the minimum change in salinity that is still percepted by the common shrimp, to a satisfactory conclu-

sion. Owing to various setbacks he was hardly successful in his attempts, and the results obtained last year underwent no significant change: the animals probably perceive changes down to 0.20 % Cl and possibly smaller. SPIEGEL conjectured that changes in the salinity of seawater are registered by means of taste perception, that is to say as a real difference in salt content.

Mr. DE BLOK, who works at Den Helder on a grant from the Netherlands Organization for Pure Research, to study the influence of lunar and tidal periodicities on the reproduction of marine animals, devoted the first six months of 1955 to examining the possibility of inducing various species of marine animals to propagate in small aquaria, as will be used in his definite experiments. He was successful as regards the worms *Polydora* sp., *Scoloplos armiger*, *Harmothoe* sp. and *Nereis diversicolor*, the molluscs *Chiton cinereus*, *Macoma balthica* and *Hydrobia ulvae*, and the sea urchin *Psammechinus miliaris*. The latter six months of the year were spent by him on further preparation of the experimental apparatus to be used. In our previous Annual Report it was stated already that the Organization for Pure Research had placed *f* 21 300.— at Mr. DE BLOK's disposal for this purpose. By means of the apparatus, which has been designed in collaboration with MR. DUK, instrumentmaker at the Zoological Laboratory, Leiden, some eight factors which vary with the lunar and tidal periodicities, will be investigated both separately and in conjuction with one another. The requisite rhythms will be evolved by mechanical devices geared to an electric clock which operates a number of rotating shafts with convenient revolution periods. These rhythms will be partly superimposed on each other. The original plan was to convert them into electric signals to be transmitted to the experimental aquaria, where they were to induce the desired rhythmical changes in pressure, light and other factors. Especially for the experiments involving varying hydrostatic pressures this electric transmission presented great difficulties. Therefore a pneumatic regulating system was adopted. For this purpose it was necessary to build a concrete pressure chamber, while also a fairly heavy compressor and expensive regulating equipment became necessary. Toward this end the Organization for Pure Research has granted an additional sum of *f* 6300.—.

In January Mr. CREUTZBERG, likewise appointed by the Netherlands Organization for Pure Research, started his investigations on the way eels find their bearings at sea, by building a shallow concrete basin with minute eel traps along the walls. By means of these it would be possible to determine whether in the dark the animals set loose in the middle would show a preference for a particular direction. In the spring and early summer months many preliminary experiments were

made with elvers caught at sea or taken from the large numbers accumulated before the sluices at Den Oever. They have not yet produced any positive results. From the outset Mr. CREUTZBERG has reserved the possibility that after its metamorphosis around the 1000 m line along the coast of the European Continent the elvers might be carried *passively* (that is by the currents), instead of actively, to within the sphere of influence of riverwater, after which the animals are supposed to be directed by hydrographic factors during their further migration. For this reason it appeared of importance to determine accurately the time of arrival and frequencies of elvers in the open North Sea off our coast. To this end, with the kind cooperation of the Netherlands Pilotage Service, widemeshed planktonnets were worked from two Dutch lightvessels, Noordhinder and Texel, the nets being handled by the crews. For a period of six months they will be hung out daily during an entire ebb and flood tide, to filter something like 10.000 m^3 of water per tidal period. In this way Mr. CREUTZBERG also hopes to find out whether elvers behave differently during ebb and flood and thus may possibly further their own transport. His plans provide for the resumption of experimental research as soon as the field work has produced sufficient indications to decide what should be the trend of the experiments. We may add here that during the autumn months Mr. CREUTZBERG also worked with silver eels which make for the open sea at that time of year. These experiments were likewise of a preliminary character and have produced no positive results so far.

Mr. WESTENBERG is engaged in theoretical considerations regarding the balance of animal populations. His review of the development of the theory of fisheries previously given in French and Dutch has now appeared in a German version (Zeitschrift für Fischerei, N.S., **4**, p. 297–314, 1955).

The data obtained this year by Mr. KRISTENSEN by buying up specimens of migratory and other species are again tabulated in this report. A remarkable point was that the southern species that as a rule arrive early were late in 1955, no doubt owing to the cold spring. Animals like pipefishes (Syngnathids), Anchovy (*Engraulis*) and the squid (*Loligo vulgaris*) did not arrive until May. Most southern species that come to these parts in spring exhibited a pronounced scarcity. This was the case with the pilchard (*Clupea pilchardus*), the John Dory (*Zeus faber*), the gurnards *Trigla cuculus* and *Trigla lineata*, the black bream (*Spondyliosoma cantharus*), the silversides (*Atherina presbyter*), and also with *Loligo vulgaris*. *Sepia officinalis* and the Syngnathids appeared in normal numbers. The southern species that are caught especially in autumn in 1955 were late, no doubt in connection with the warm autumn weather of that year. Examples were *Scyllium canicula*, *Mustelus vulgaris*.

Species	*Sex; Size (cm)*	*Locality* (*ST means buoy on the Silverpit-Texel route*)
MIGRANTS SUPPOSED TO HAVE ENTERED THE NORTH SEA VIA DOVER STRAIT		
Cetorhinus maximus	± 250	Inschot (W. Waddensea)
Scylliorhinus stellaris	♀ 51; ♀ ± 45	Tea Kettle Hole; Callantsoog, 13 m
Raia brachyura	33–108	Texel Hole-Tea Kettle Hole
Raia montagui	32–69	Texel Hole-St 2- N. of Terschelling
Hippocampus europeus	♀; ♂	Texelstroom (W. Waddensea)
Spondyliosoma cantharus	29.5; 27; 21.5	near ST 4
Trigla cuculus	23–31	Texel Hole–ST 1
Solea lascaris	♀ 26.5; ♀ 24	Texel Hole; ST 3
MIGRANTS SUPPOSED TO HAVE ENTERED THE NORTH SEA VIA THE NORTHERN ENTRANCE		
Cetorhinus maximus	♂ 351	ST 4
Scombresox saurus	34–37	stranded between Egmond and Texel
Phycis phycis	37; 53	58°20′ N, 2°15′ E, 104 m; 58°20′ N, 3° E, 108 m
Brama raii	51–63	58° N 3′ E; Zeeland (2 spec.); 54°25′ N,2°25′E; 58°20′ N, 2°15′ E; Zandvoort; Zeeland; Huisduinen
NORTHERN FISHES, RARE IN THE S. NORTH SEA		
Raia radiata	♀♀ 40, 45, 46, 48	Pit 3–4 buoy 44 m (3 spec.); ST 4
Onos cimbrius	25; 19; 22; 22	West Hole; off Callantsoog; N. of Terschelling, 26 m (2 spec.)
Anarhichas lupus	30–36	Texel Hole–ST 2
Hippoglossus hippoglossus	27	ST 4
FISHES, WHOSE DIRECTION OF MIGRATION IS UNCERTAIN		
Petromyzon marinus	60–66	Zuiderhaaks; River Maas (30 spec.)
Merluccius merluccius	24.5	Texel Hole area
Raniceps raninus	18; 13; 25	Zuiderhaaks; Camperduin; Zuiderhaaks
Acipenser sturio	185	NW of L V Terschellingerbank, 36 m
Capros aper	15.2	near PE 4 buoy (= 54°02′ N, 6°51′ E), 31 m
Pagellus centrodontus	25.8	54°18′ N, 5°21′ E
Labrus berggylta	5	Texelstroom (W. Waddensea)
Crenilabrus melops	18.5; 16.5; 20	Zuiderhaaks; Egmond; Huisduinen
Scorpaena dactyloptera	17.5; ?; 15; 19; ± 10	Westhole; Texel Hole; Black Bank; Texel Hole Wadden Sea
Zeugopterus punctatus		ST 4
EVERTEBRATES, "RARE" IN THE AREA OFF DEN HELDER		
Nephrops norvegicus		Texel Hole area–ST 4
Todarodes sagittatus	♀ ± 40	stranded near Petten
Todaropsis eblanae	♀ 9.6; 10.3	ST 2
Loligo vulgaris		mostly from coastal waters (no data collected in July–Oct.)
Loligo forbesi		mostly from Texel Hole area (no data collected in July–Oct.)
Sepia officinalis		mostly from coastal waters and Wadden Sea
Octopus vulgaris	♀ 7; ♂ 14; 5 ♀♀ 13–15	Texel Hole area – ST 3
Eledone cirrhosa		Texel Hole area – ST 4
Pontobdella Vosmaeri	♀	SW of Texel Hole

Numbers per month											
J	F	M	A	M	J	J	A	S	O	N	D
—	—	—	—	—	—	1	—	—	—		—
—	—	1	—	—	1	—	—	—	—	—	—
1	3	—	2	1	—	1	—	—	—	1	—
19	7	6	2	2	1	3	2	—	4	1	4
—	—	—	—	—	—	—	1	1	—		—
1	—	—	—	—	—	1	—	—	—	—	1
2	—	—	—	—	3	2	—	—	—	—	
1	—	—	—	—	1	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	1	—
—	—	—	—	—	—	—	—	—	—	—	> 100
—	—	—	—	—	—	—	—	—	—	1	1
—	—	—	—	—	—	—	—	—	1	4	3
—	—	3	—	—	1	—	—	—	—	—	—
1	—	1	—	—	—	2	—	—	—	—	—
—	—	—	6	1	1	—	—	—	—	—	—
—	—	—	1	—	—	—	—	—	—	—	—
—	—		—	1	30	—	—	—	—	—	—
—		—	—	—	1	—	—	—	—	—	—
—	—	—	2	1	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	1	—
—	—		—	—		—	1	—	—	—	—
—	—	—	—	—	—	1	—	—	—	—	—
—				—	—	—	—	—	—	1	—
—	—	1	—	—	—	—	1	1	—	—	—
1	—	1	1	—	—	—	—	—	—	2	—
1	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	2	—	2	—	—	2
—	—		1	—	—	—	—	—	—	—	—
—	2	—	—	—	—	—	—	—	—	—	—
—	—	—	—	5	32	?	?	?	?	—	—
—	—	—	—	—	41	?	?	?	?	100	7
2	—	—	—	17	216	29	5	—	—	—	—
—	—	—	—	—	—	—	1	—	—	—	6
5	1	—	—	—	—	—	—	—	—	1	—
—	—	—	—	—	—	—	1	—	—	—	—

Raia montagui, *R. brachyura* and *Octopus vulgaris*. Of these species only *Octopus vulgaris* was numerous, the others were definitely scarce. Of the Atlantic species which enter the North Sea particularly from the North, fairly normal numbers were observed of the bream (*Brama raii*), the skipper (*Scombresox saurus*) and the squid *Loligo forbesi*. A specimen above 2.5 m long of the basking shark (*Cetorhinus maximus*) was observed by Mr. KRISTENSEN in the Inschot (Waddensea) in July, and another, 3.5 m in length, was caught northwest of Vlieland in the North Sea in November. The latter was without gillrakers, a fact of interest in connection with the supposed periodical loss of these, see PARKER and BOESEMAN, Proc. Zool. Soc. London, 1954. Among the species of fish with a northern distribution, *Anarhichas lupus*, the catfish, was somewhat more numerous than was the case in the last few years (though still less common than before the war), whereas haddock, which before the war had practically disappeared from the Dutch coast, were again caught this year in small numbers off Den Helder (in April).

It is worth mentioning that in December more than 100 *Scombresox saurus*, the Oceanic skipper, got stranded on the beach between Egmond and Den Helder.

A second point of interest is that in August and September a specimen of the grey seal, *Halichoerus grypus*, was observed a few times near Den Helder, off Texel and on tidal sands outside the Marsdiep. In the latter area it was filmed by Mr. H. Th. DE BOOY, Honorary Secretary of the Netherlands Humane Society, who kindly placed the reel at our disposal. The animal shared its resting-place with ordinary seals, but was striking through its length, which was about 3 metres. The only certain record of this species on our coast hitherto consisted in a lower jaw found by VAN GIFFEN in a Groninger mound from about the 10th century.

Finally, it should be mentioned that oviposition of a specimen of the leech *Pontobdella vosmaeri* occurred in the aquarium.

The hydrographic work under the direction of Mr. POSTMA made good progress, as will appear from the following.

In the first 3 months of 1955 Mr. SOERIAATMADJA continued his investigations mentioned in the previous Annual Report, which concerned the influence of the North Holland Canal on the water of the Wadden Sea near Den Helder. Mr. SOERIAATMADJA found that only in a very narrow belt of but a few hundred metres width along the seawall of Den Helder does the water from the Canal have a fertilizing influence on the water of the Wadden Sea; indeed, this influence is only clearly perceptible around slack low tide. The intensive mixing caused by the strong currents apparently takes up the fresh water transported to sea so quickly that after a single ebb and flood tide its presence can

hardly be demonstrated, neither as regards its effect in decreasing salinity nor in its fertilizing influence.

In January Mr. AL KHOLY of the Marine Biological Laboratory at Ghardaqua on the Red Sea wound up the research items undertaken by him in 1954. He left for Kiel and Copenhagen in February.

In cooperation with Mr. M. ROFSSINGH of the Government Institute of Fisheries Research a study was made in February and March of the distribution in the North Sea of water transported from the Wadden Sea through the Marsdiep. To this end both the "Anthonie van Leeuwenhoek" of the Institution of Fisheries Research and the "Max Weber" made two trips, the former ship dealing with the North Sea area, the latter with the Western Wadden Sea. Water samples were taken near the bottom and the surface at 40 stations in all. Of these samples, apart from salinity, a large number of chemical factors were determined which might be expected to show differences between the Wadden Sea and the North Sea. From the analyses it appeared that the presence of the Wadden Sea water could be demonstrated rather far to the South along the coast, probably as far as Petten. All this water is then transported to the North by the residual current in the North Sea. It thereby does not hug the coast of Texel at all closely, but is quickly transported a long way from the island. The investigation will be continued in the early part of 1956.

For six weeks in the summer Mr. P. EVERHARDUS, a student of chemistry of the Vrye Universiteit, Amsterdam, and a pupil of Prof. P. GROEN, studied tide-rips. For this piece of research he used a self-registering salinity-meter temporarily placed at our disposal by the Royal Netherlands Meteorological Office. Tide-rips mark the dividing line between two water-masses, which may exhibit large differences in salinity, temperature and other properties. From a spatial point of view, they are dividing-planes rather than dividing-lines and Mr. EVERHARDUS's main point of investigation was to determine the exact location of these dividing-planes between the bottom and the surface. He further tried to find out how fast the surface water flows to the tide-rip under certain conditions, and whether this occurs on one or on both sides. Finally, he tried to form an idea of the stability of the dividing planes at an increasing rate of flow of the tidal current. The results cannot yet be set forth here.

As in former years assistance was given to Rijkswaterstaat, section Hoorn, by carrying out a number of determinations of the percentage of suspended material in the New Harbour. Also in other respects the composition of the water of this harbour was studied, as will appear further on.

As in 1954, a number of phosphorus determations were made of

water samples collected in the Easter Scheldt. In 1956 this form of assistance to Dr. P. KORRINGA's productivity studies will not have to be continued, as he now has his own Laboratory for Oysterculture at Wemeldinge. Miss H. FAVERY DE JONGE, analyst of this laboratory, spent a week at Den Helder to become acquainted with the chief determinations of seawater that are of importance for the above mentioned productivity studies.

Also other institutions sent workers to Den Helder to get acquainted with the methods of chemical-hydrographic investigation. They were: Mr. TIMMERS of the Government Institute for the Purification of Wastewater, Section Dollart, Baflo, Miss Le Cosquino de Bussy of the National Defence Organization T.N.O., Mr. DE VRIES of the Government Institute of Fisheries Research, IJmuiden, and Mrs. VAN OORDE- DE LINT and Mr. PEELEN of the Research Team for Biological Investigation of the Big Rivers, Amsterdam. For the last mentioned study, water samples were taken by this Research Team at regular intervals in the course of a year. They were analysed at Den Helder by Mrs. VAN OORDE and Mr. PEELEN after they had become acquainted with the methods. Finally, we should mention that in April some research workers of the Bataafse Petroleum Maatschappij called in our assistance for the collection of recent plankton and sediments in the Wadden Sea. For this purpose the laboratory and the Max Weber were used for a few days.

In addition to the work mentioned above and the assistance given to other institutes Mr. BEKE continued his analyses of suspended material.

Mr. POSTMA himself spent considerable time on preparing for publication the data collected at Hamburg and Naples in 1954. He further gave his attention to the bottom samples collected over a number of years during the biological inventory of the Wadden Sea. These sedimentological data, which are also of interest in themselves, were likewise prepared for the press. In the last few months Mr. POSTMA has also undertaken to work out the data on oxygen, alkalinity, pH and phosphate of the Snellius Expedition. Also part of the temperature and salinity values were taken into account to obtain an approximate picture of the currents between bottom and surface. It is hoped he will be able to terminate this work in the first six months of 1956.

The following persons spent short terms at Den Helder: Mr. H. J. DE JONG, Leiden, to study jaw movements in live fish; Mr. W. J. R. LANZING, Utrecht, to read up references in our library; Mr. A. ROOSENSCHOON, Utrecht, to make observations on cockles digging themselves into the sand; Mr. J. H. STOCK, Amsterdam, for studying parasites of Lamellibranchs; and Mr. W. S. VOLKERS, Leiden, to obtain specimens of the developmental stages of *Psammechinus miliaris*. Also this

year some assistance was given to the Netherlands Educational Films.

The number of students who attended the summercourses was 23, of whom 18 came from Leiden, 2 from Utrecht, 2 from Groningen, while also Mr. SOERIAATMADJA, the Indonesian analyst, attended the course. The courses were given jointly by all the members of the staff. They were held from 13th to 25th June and from 27th June to 9th July.

From 13th to 23rd April Mr. PUNT, with the assistance of Mr. VAN NIEUWENHOVEN and Mr. PARMA, gave a physiological course for biologists from Amsterdam, which was attended by 13 persons. Moreover, the first and second years students from the Vrije Universiteit, Amsterdam, accompanied by Mr. ANTHEUNISSE and Mr. JOOSSE, visited the Zoological Station from 23rd to 28th May and from 31st May to 4th June, while a group of 20 second year students from Utrecht, conducted by Mr. KIPP, paid us a visit from 6th to 8th and from 9th to 11th June.

The total number of man-days for individual workers and summercourse participants amounted to about 1380. The average for the years 1947–1955 is approximately 1110.

Among the visitors from abroad who paid us brief visits were: Prof. W. E. ANKEL, Giessen (Germany), Prof. TAKEO IMAI, Sendai (Japan), Prof. and Mrs UMBERTO D'ANCONA, Padova (Italy), Dr. and Mrs. PETER DOHRN, Naples (Italy), Prof. D. DAVENPORT, Santa Barbara (California), Prof. F. HAXO, Scripps Institution of Oceanography, LA JOLLA (California), Dr. A. ALEEM, Cairo (Egypt), T. K. RUEBUSH, Office of Naval Research, U.S. Navy, London (England).

The maintenance of the building in 1955 required no large-scale expenditure. The caretaker's living-room was redecorated; an internal telephone-system was installed. As Mr. DE BLOK will take over for his own use the new cellar under the net store an open shed was built to store the wicker bottles, tins, crates, etc., which are used in forwarding study specimens and other material.

It should be put on record that the collection of animals from the Southern North Sea, which had been stored away in the National Museum for Natural History at Leiden during the war, was brought back to Den Helder towards the close of 1955. The director of the Museum deserves the expression of our sincere gratitude for his inexhaustible patience.

The Station's equipment was supplemented by the purchase of a stereomicroscope of *f* 1850.— and a Philips stroboscope of *f* 550.—. We also want to place on record that the refrigator was at long last equipped with running cooled water. This improvement contributed to the successful accomplishment of Mr. JANSSEN's *Littorina*-experiments.

The "Max Weber" was equipped with the electric lighting to which we had been looking forward for years. The current is generated by a dynamo mounted on the mainshaft. The skipper, Mr. VAN BREDA, thoroughly overhauled the ship. It spent a smaller number of days at sea than in the past years, which is to be attributed to the kind of research undertaken this year. The maintenances of the two rowing-boats required no special measures.

On 15th May Mr. DIJKSTRA entered the service of the Zoological Station in the newly created function of managing assistant. The purpose of this appointment is to relieve especially the Director of many administrative and organizing duties. On 1st December Mr. VAN BREDA, skipper and caretaker, was released from his function at his own request. On 1st April Miss G. WATTIEN joined the staff as student-analyst, taking the place of Mr. G. VAN DER WAL.

In April Mr. VERWEY attended an International Conference on Marine Biological Stations in Rome. On that occasion he also spent some days at the Zoological Station at Naples. Early in the autumn he attended the Meetings of the British Association for the Advancement of Science at Bristol. He took that opportunity to visit the catching-grounds of elvers on the river Severn, the Laboratories for Shellfish Research at Conway (Wales) and Burnham-on-Crouch (Essex), and the new Marine Biological Laboratory on Anglesey off Bangor (Wales), a small, very modern Institute with admirable facilities and great possibilities.

The condition of the library remains quite satisfactory since it has become possible to give more time to it than in former years. The Organization Het Natuur- en Geneeskundig Congres donated a sum for the purchase of Russell's fine work "The Medusae of the British Isles". Also to other donors of books and separates we wish to express our sincere gratitude. A small number of books were bought, also a few series of periodicals, among which the Lincolnshire Naturalist, which was hitherto not available in this country. The binding of books and periodicals made steady progress. The need for more library space is gradually becoming pressing.

The supply of study materials to Universities, etc. brought in *f* 4990.03, against which must be set costs amounting to *f* 4512.17. This amount includes expenditure for wicker bottles and other packing materials, preserving solutions, etc., totalling *f* 1257.27.

The Government subsidy amounted to *f* 93 500., of which *f* 77 534.75 was paid to salaries. The other expenses are given in the Statement which follows.

The Government moreover refunded last year's deficit of *f* 2260.35, while at the end of the year the Government allocated a sum of

STATEMENT OF INCOME AND EXPENSES

Income		*Expenses*	
Government grant	*f* 93 500.—	Salaries	*f* 72 113.96
Grant from Roy. Netherl. Bot. Society	,, 100.—	Auditing	,, 600.—
Contribution to salaries from Reservefund	,, 500.—	Contribution to National Insurance	,, 5 420.79
Study Materials	,, 4 990.03	Rates, taxes, and insurance	,, 1 762.20
Sickness Benefit	,, 46.29	Lighting, heating, telephone	,, 5 635.07
Contribution to Library from the Society	,, 300.—	Buildings and furniture	,, 2 455.15
Deficit	,, 4 175.96	Ships and nets	,, 2 569.70
		Instruments, glass utensils, chemicals	,, 3 761.08
		Fitting shop	,, 623.32
		Study Materials	,, 4 512.17
		Postage, freight packing, writing and drawing materials	,, 1 967.78
		Library	,, 1 365.90
		Travelling expenses	,, 634.22
		General	,, 190.94
Total	*f* 103 612.28	Total	*f* 103 612.28

f 21 000.— for urgently needed improvements, which will be mentioned in the Report for 1956.

If one considers that the Government Department settles pension payments direct with the National Pension Fund, and that through the Netherlands Organization for Pure Research they contribute another *f* 15 000.— for the salaries of two research workers in addition to considerable grants for their equipment, the actual overall expenditure for the Zoological Station should be put at something like *f* 125 000.—. It is to be expected that in the years to come this amount will increase considerably, a state of affairs which is commonly met with in all kinds of research institutes. This period of civilization might appropriately be called the Era of Scientific Research.

A few remarks should finally be added about the new harbour. In the reports for 1952 and 1953 something was said about its consequences for the Zoological Station, particularly as regards the supply of seawater of a good quality. Now that the new harbour is largely finished, it seems fitting that we should briefly revert to this question. The old outer harbour (Buitenhaven) has now been dammed off both on the landside and in its outer end. From the old harbour opposite the locks of the Naval Dockyard ships can now reach the sea through

the wide new harbour. The seawater therefore enters the old harbour through the new one. Through the latter the fresh water of the North Holland Canal is discharged. A counter current of salt water is developed along the bottom, so that in front of the Zoological Station, where our seawater pipeline cuts across the dike, salt water of a good quality is present at all times. In winter it is pumped up and used for the aquaria. In the summer months the enormous amounts of sediment on the bottom cause a lack of oxygen in the lower water layers. A waterboat then brings in supplies from the Marsdiep. The cost of this transport, which most years takes place from May to October, is borne by the Royal Navy. As a result of excessive sedimentation the depth of the harbour has greatly decreased. For this reason an extra vertical length of piping has been welded to the inlet, so that its mouth now lies 7 metres below the surface instead of 9.

Den Helder, February 1956
J. Verwey

AN EXPERIMENTAL STUDY ON THE TIMING OF BREEDING AND MIGRATION IN THE THREE-SPINED STICKLEBACK

(GASTEROSTEUS ACULEATUS L.)

by

B. BAGGERMAN
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Annual periodicity in reproductive behaviour is a common phenomenon in many Vertebrate species. Breeding is often correlated with migration to and from a certain area. The present investigation deals with the causation of the timing of both types of behaviour.

PART I

THE TIMING OF THE BREEDING SEASON

I. INTRODUCTION

Reproductive cycles are evident in most species of Vertebrates. Not only animals living in temperate and sub-arctic regions but also many species living in the tropics possess a well defined annual cycle. The most striking problem in seasonal breeding is the fact that its incidence is so amazingly punctual each year, while seasonal climatic conditions are subject to great variations. The latter might give rise to the assumption that intrinsic factors are responsible for the timing of reproduction. However, though there is evidence that internal physiological factors influencing the regulation of annual breeding do exist, it is inconceivable that the phenomenon can be wholly controlled by them. Therefore, it must be assumed that external factors play a rôle, either in co-operation with internal factors or not. In the following discussion the question of breeding in the tropics will not be considered because the relatively uniform external conditions make seasonal reproduction in this region a problem in itself.

In temperate zones there is one climatic variant that occurs punctually each year, namely the increase in day length. Rowan was the first to approach experimentally the problem of the influence of the daily photoperiod on reproduction. Since then a large number of investigations have been made on the rôle of day length in the timing of seasonal breeding in various species of Vertebrates. But it was gradually discovered that, though the duration of the daily photoperiod was important in some species, it had no or little effect in other species. Therefore, other external conditions like temperature, quality and quantity of light, food etc. have been studied with respect to their possible influence on the timing of seasonal breeding. Temperature proved to be particularly important in some species, mostly cold blooded animals.

The stimulating results obtained from investigations on the controlling influence of external factors soon gave rise to the conclusion that

usually only external conditions play a rôle in the timing of the reproductive season. However, some investigators had serious objections to this premature generalization and held that an intrinsic rhythm was the main cause of annual breeding. Though it is generally realized now that such a rhythm cannot be the sole factor causing sexual periodicity, investigations made on this subject and also observations on reproduction in animals transported from one hemisphere to the other, showed that in many animals intrinsic factors which influence the timing of the breeding cycle must be present. It is generally held now that in most animals the timing of the breeding season is controlled by a close co-operation between external and intrinsic factors.

In this brief survey no literature has been cited, but references can be found in the discussions at the end of each chapter in the present study, while the following papers give reviews or literature: Marshall, 1936, 1937, 1942; Bissonnette, 1938; Rowan, 1938; Bedford and Marshall, 1942; Beach, 1948; Bullough, 1951; Hoar, 1951b, 1955; and Memoirs of the Society for Endocrinology No. 4, 1955.

In the present investigation the timing of the breeding season was studied in the three-spined Stickleback. Craig-Bennett (1931) studied this subject in the same animal and came to the conclusion that day length had no influence, but that temperature was the main factor controlling reproduction. However, experiments of van den Eeckhoudt (1946) and N. Tinbergen (pers. comm.) showed that light did affect the rate of gonadal development in the early months of the year. This controversy made a new and more detailed investigation of the same subject necessary.

In most studies on the regulation of reproduction histological criteria (stages of spermiogenesis or oogenesis) have been used to obtain information on the effect of external conditions on gonadal development. In many cases this method has proved to be very valuable, but there is one disadvantage that is usually left out of consideration. Actual breeding is not only characterized by gonadal development or the presence of ripe sperm and ova, but also includes the ability to carry out reproductive activities necessary for the fertilization of the eggs. That the use of histological criteria might give rise to wrong interpretations can be illustrated with the following example. Craig-Bennett (1931) found that in the male Stickleback in mid-winter the gonads already contain ripe sperm that are able to fertilize eggs. However, at this time of the year the animals are not yet able to perform reproductive behaviour. This is due to the fact that the interstitium, though present, has not yet started to develop and secrete gonadal hormones. Thus, it is clear that in this case the presence of ripe sperm

may lead to the wrong conclusion that the animals were mature. Knowledge of the state of interstitial development would have made it clear that these animals were not yet mature. Nevertheless, even in case the interstitium had also developed this would not have been sufficient proof that the animals were ripe, for the presence of a fully developed interstitium (as far as can be judged from histological preparations) does not yet mean that it is functionally active, i.e. actually secreting hormones that are necessary for the performance of reproductive behaviour.

Therefore, by using histological criteria only, one can never be completely sure whether an animal is mature or not. This was the main reason that in the present study reproductive behaviour was used as a criterion for the attainment of maturity. In this case one is always sure that the animals are actually in breeding condition. The ideal method would have been to make histological preparations of pituitary and gonads in combination with the study of behaviour, but this was impossible in the present study.

In the investigation attention was paid not only to factors causing the onset of the reproductive period, but also to factors responsible for the termination of this phase. Until now very little work has been done on the latter subject, though it is at least equally important as the regulation of the onset of breeding. It also seems to have escaped the attention of many workers in the field that in the case of spring breeders, the onset of breeding is not only determined in the period between mid-winter and spring, but that its timing starts as soon as gonadal recrudescence begins after the end of the previous reproductive period. In many species, for example, spermiogenesis and oogenesis begin very shortly after breeding. In birds it is a well known fact that there is a revival of sexual activities in late summer, while, for example, in the case of the Stickleback, Craig-Bennett (1931) has shown that spermiogenesis and oogenesis start already in July and August, i.e. almost immediately after the end of the breeding season. Turner (1919) found the same for the beginning of spermiogenesis in the Perch *[Perca perca]*. Therefore, in the present investigation the influence of external and internal factors on the gonads have been studied at various times in the annual reproductive cycle.

In many investigations on the influence of external factors on reproduction extreme conditions have been used, such as complete darkness or continuous light during 24 hours daily. But it is a completely different question whether an animal responds to either 24 hours light or complete darkness, or whether it is able to react to much smaller differences such as occur in nature. This was one of the main reasons that in the present study only photoperiods between 8 and 16 hours were

used, corresponding with the shortest and the longest day lengths occurring in nature at the latitude of Holland. Also the temperature range that was used corresponded to that to which the animals are usually exposed the year round in nature, namely from 4–20° C.

The subjects studied were those which could mostly be applied to circumstances in nature. Thus, the effect of various external conditions on all phases of gonadal development was studied, and the function of this effect in the timing of breeding in nature is discussed. An attempt was also made to find factors which might be of critical importance for breeding. The duration of the breeding period under different external conditions was studied and also the factors causing the termination of this phase. Attention was paid to possible differences between the responses of males and females and between those of under-yearling and adult fish.

II. MATERIAL, METHOD AND TECHNIQUE

1. MATERIAL

The animals used were caught at various places in the north of Holland, namely at Oostzaan, Den Helder, Zoutkamp and Termunten. The fish belonged to three different races distinguished by Bertin (1925), namely *Gasterosteus aculeatus forma trachura*, *forma gymnura*, and *forma semi-armata*. The races are distinguished according to the number of bone plates on the sides of the fishes and to the presence of a caudal crest (formed by very small plates). The *trachura* are covered from pectoral fin to tail with plates and also have a caudal crest. The *gymnura* have only a small number of plates near the pectoral fin and no caudal crest, while the *semi-armata* are partly covered with plates and also have the caudal crest.

In preliminary experiments it was shown that the three races responded in the same way to similar external conditions. For example 10 *trachura*, 10 *gymnura*, and 8 *semi-armata*, all coming from the same group caught in nature, were exposed to a day length of 16 hours and a temperature of 20° C. The *trachura* matured between 16 and 22 days after the beginning of the experiment (average response-time 19 days), while the corresponding figures for the *gymnura* were 16–20 days (average duration 18 days) and for the *semi-armata* were 15–21 days (average duration 18 days). There was no statistical difference between the duration of the response-times in the three groups. Results in other experiments in which different external conditions had been used were similar to the above. Therefore, in all experiments the three races were used at random and usually all races were represented in each experimental and control group.

2. BEHAVIOUR AND CRITERIA FOR THE ATTAINMENT OF MATURITY

The 3-spined Stickleback lives in the sea during winter and migrates to fresh water in the early months of the year. The breeding season in Holland begins around the middle of April and lasts until the end of June or the beginning of July. At this time the animals start to migrate back to the sea. For further information on migration see Part II, Chapter II.

During the breeding season (April until July) the male builds a nest on the bottom of ditches or other shallow waters. Before building the nest the male occupies a territory and defends it against intruders. Nest-building is started with the digging of a shallow pit into which the animal brings small fragments of plants that are tightly glued together by a substance secreted from the kidneys. When the nest is completed it has a well defined entrance. The completion is indicated by the male creeping through the nest. A ripe female entering the territory of a male with nest is courted by the male and led to the nest by means of a series of mutual courting activities. The female creeps into the nest and deposits her eggs after which she is chased away by the male. The male also creeps through the nest to fertilize the eggs and takes further care of them afterwards. During the time the eggs develop (about 8–10 days, depending on the temperature) the male regularly makes fanning movements in front of the nest entrance in order to supply the eggs with oxygen-containing water. When the young have hatched they usually remain in the nest pit during the first few days, but gradually spread over the whole territory and outside. When the young have disappeared the male usually starts to build a new nest.

The reproductive behaviour of the male Stickleback can be divided into three phases, namely the nestbuilding, the sexual and the parental phase. The nestbuilding phase ends when the nest is completed. According to VAN IERSEL (1953) the nest is finished when the male creeps through the nest. Since it was impossible in the present study to watch the animals regularly, the nest was considered to be finished when it had a well defined entrance and was tightly glued together. With some experience it is easy to determine the end of the nest-building phase. When the nest is finished the animal enters the sexual phase in which he is always willing to lead a ripe female to the nest and to fertilize eggs. After fertilization of the eggs the male enters the parental phase in which he cares for the eggs and young. When no ripe female comes into the territory the male usually builds a new nest after some time (one week or longer). For details on the reproductive behaviour see TER PELKWIJK and TINBERGEN (1937) and VAN IERSEL (1953).

In the present investigation nestbuilding was used as criterion for the attainment of maturity by the male, for as it has been mentioned above, the animal enters the sexual phase when the nest is completed. This criterion proved to be very appropriate for three reasons. In the first place when a male has completed the nest it is always willing to lead a ripe female to the nest and to fertilize the eggs. Therefore it can be considered to be fully mature. In the second place the majority of nests is finished within 24 hours, though usually within a shorter time. This means that with regard to the date of maturity a mistake of only one day can be made. In the third place there seems to be a kind of "all-or-none" law in nestbuilding.The animals either build a nest or they do not. Nests that seem to be only partly finished will be finished within a short time.

In the female the state of maturity was determined by introducing her into the territory of a male with a nest. When she deposited eggs in the nest she was considered to be mature. A female that is not fully ripe will not enter the nest, though she may do so only a couple of hours later. In case there was no male available with a nest, the female was stripped. In a fully ripe female this can be very easily done without harming the animal. However, females that are not fully ripe cannot be stripped, though this may succeed only a few hours later.

3. STORAGE OF THE FISH AND EXPERIMENTAL CONDITIONS

After the fish had been caught they were transported to the laboratory and stored either in a large basin outside the building or in large tanks in the aquarium-house. The basin was about 9 m long, 4.5 m broad and 0.7 m deep. It was situated on the north side of the building, but received sunshine during part of the day. A thermograph registered the temperature at about 25–45 cm from the bottom.

The large tanks in the aquarium-house were 120 cm long, 60 cm broad and 60 cm high. They were made of concrete with a glass window in front and were also covered with glass. Sunshine could not penetrate into the tanks, but the light intensity in the aquarium-house was so high that very often screens had to be used in order to prevent the growth of green algae. The day length in the aquarium-house was similar to that in nature, but the temperature was usually somewhat higher, particularly in winter.

In fig. 1 the natural day length in the course of the year is given together with the temperature of the outdoor basin and the aquarium-house. The data represent the mean monthly day lengths and temperatures. During a certain period of time the temperature was also measured in a shallow ditch in the neighbourhood of the laboratory

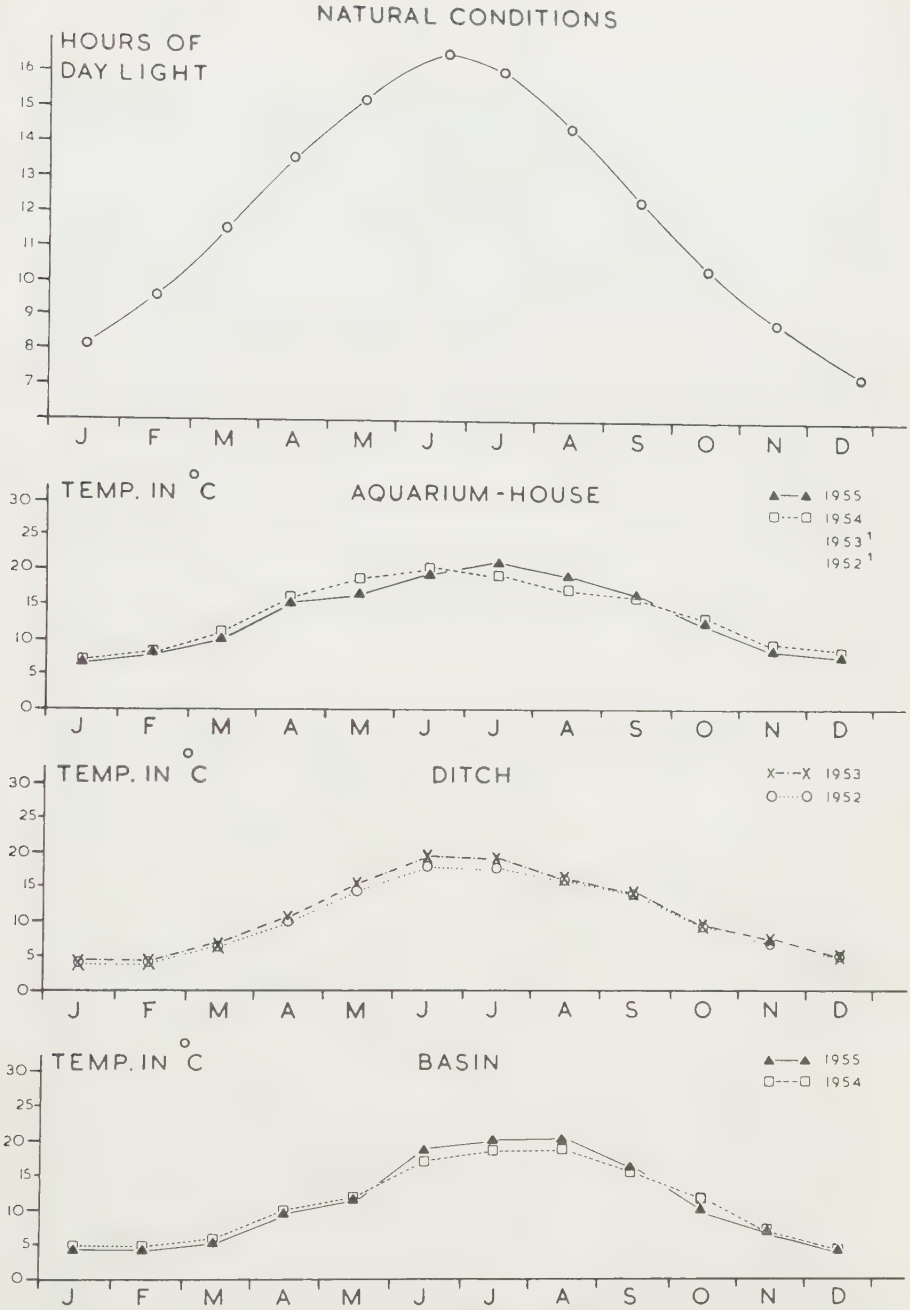

Fig. 1. Day length and temperature conditions of different years in the aquarium-house, in a shallow ditch, and in the basin outside the laboratory. The points indicate the monthly averages of day length and temperature.

[1] The figures for 1952 and 1953 follow about the same curve as those of 1955 and 1954.

and it can be seen in fig. 1 that the temperature in the basin is comparable to that in the ditch. Therefore, the conditions in the basin were regarded as natural conditions.

The majority of experiments (those from July 1953 until 1956) were carried out in small rooms in which the temperature could be regulated with great accuracy. The daily fluctuations never exceeded 0.4 degrees C. Four of such rooms were available; in two of them the temperature could be regulated from room temperature up to 40° C, while in the other two the temperature range was from 0–40° C. The first two rooms were about 320 cm long, 220 cm broad and 237 cm high, while the latter rooms were 240 cm long, 195 cm broad and 237 cm high. All four rooms had white painted walls.

The temperature rooms were illuminated with ordinary bulbs. In the largest cells one bulb of 150 Watt was suspended in the middle of the room and one bulb of 75 Watt at either end. The aquaria in which the animals were kept were placed along the walls and the light intensity that fell on the water surface was in all tanks 25–30 f.c. In the smaller cells one bulb of 150 Watt in the middle of the room was sufficient to provide a light intensity of 25–30 f.c. at the water surface of all tanks.

In a few experiments made before July 1953 (in the old laboratory) other rooms were used. In these cases the light intensity was higher than 25–30 f.c., namely about 50 f.c. Also a different kind of illumination was used (fluorescent tubes). But in Chapter IV it will be shown that the different types of light used in the two laboratories had the same effect on maturation of the fish.

In the introduction it has already been mentioned that the ranges of day lengths and temperatures that were used never exceeded those occurring in nature. This meant that the day lengths were never shorter than 8 hours or longer than 16 hours, while the temperature range was from 4–20° C. The day lengths in the temperature cells were regulated by electric clocks.

In two temperature rooms conditions were kept constant during the three years of experimental work. In one of these rooms the day length was 8 hours and in the other 16 hours, while the temperature was 20° C in both cells. This was done for several reasons. In the first place preliminary experiments had shown that these two conditions were very appropriate for giving information on the level of gonadal development of animals exposed to these conditions. In the second place it was the most economical way to make large series of experiments because the fish could be exposed to these conditions at any time.

The two remaining rooms were mainly used for experiments with low temperatures. When necessary the animals that had had a cold

treatment could be transferred to one of the other two rooms for exposure to high temperatures and the former cells could be used again for other experiments in which low temperatures were required.

The tanks in which the animals were kept in the temperature rooms were 60 cm long, 35 cm broad and 35 cm high. In these tanks a maximum of 7 adult fish was kept; that is about 10 l of water per fish. In case more than 7 animals had to be used some were placed in other aquaria where again the amount of water per animal was at least 10 l. Care was taken that the amount of water per fish was always about the same in the experimental as well as in the control groups. This means that a possible crowding of the tanks (which may have influenced nest-building) was the same in both groups. When under-yearling fish were used a maximal number of 15 animals (depending on their length) was used per tank.

As has been said nestbuilding was used as criterion for the attainment of maturity in the males. For this reason the tanks were adequately supplied with nesting material. Males about to start nestbuilding first occupy a territory from which all other fish are chased away. The latter are usually driven into a corner where they are more or less left at ease. However, as soon as a male had finished his nest he had to be taken out of the tank because his aggressive behaviour usually prevented nestbuilding in other males living in the same tank. The tanks were examined twice a day and in this way a possible inhibition of nestbuilding in other animals by males with a nest was excluded as much as possible.

In the 3-spined Stickleback there are indications that the presence of a small number of other fish in the same tank may stimulate reproductive behaviour, while the latter is suppressed by overcrowding. However, in all experiments the tanks were far from overcrowded and, therefore, suppressing of reproductive behaviour cannot have played a rôle. No data are available on the effect of a possible stimulation of sexual behaviour by the presence of other fish. If this were true it might seem that the last animals to build a nest were in an unfavourable condition compared with the first animals. However, in all cases the animals could see other fish in adjoining aquaria to which they reacted in almost the same way as to animals in the same tank. Therefore, the last animals to build a nest were never visually isolated from other fish.

In females there do not seem to be indications that the presence of other males or females might stimulate maturation. In all cases the females were kept in the same tank as other females and males and also could see fishes in adjoining aquaria.

Care was taken that the animals were always well fed. Usually live

food was given (*Daphnia*, *Tubifex*) but in case this was not available the fish were fed with cooked ground liver which they ate quite well. All tanks were provided with green vegetation, while the bottom was covered with sand and kept clean from decaying food. If necessary the water was aerated to provide a sufficient supply of oxygen.

4. STATISTICAL METHOD

In many experiments it was necessary to test whether the responses of one group of fish was different from that of another group. In most cases only a limited number of animals could be used because there was no space for more; usually a group consisted of 3–10 animals. It was not known whether the responses would be distributed according to normal distribution. In this case the best method that can be used is the test of WILCOXON (1945); see also MANN and WHITNEY (1947).

From the data of both groups the value of U can be calculated and the P belonging to this value of U indicates whether the difference between the two groups was significant. In the experiments a value of P equal to or smaller than 0.05 was considered to indicate a significant difference.

A. FACTORS INFLUENCING THE ONSET OF THE BREEDING SEASON

At the beginning of this part of the paper some introductory remarks must be made. It will be seen that in the various experiments animals were used that had previously lived under different conditions. Thus in some experiments fishes were used that had been caught in their natural habitat, while in others the animals had been maintained under artificial conditions. Also the time of the year in which the experiments were made played a rôle. This generally meant that the phases of gonadal development in which the animals were at the beginning of the experiments varied between the various experiments. In later chapters the existence of such phases will be proved experimentally. However, for a better understanding and also to make comparison between the various experiments easier, the phases of gonadal development in which the animals were at the beginning of the experiments will be mentioned in the description of each experiment.

In order to give the reader some understanding of the way in which the phases were determined, a very brief description of the various phases will be given here. It must be strongly emphasized that these are physiological phases which are defined by their response to a number of experimental external conditions used in the investigation.

By response is meant that under the influence of a particular condition the gonads developed to the next phase or further. Details on the experimental basis for the distinction between the various phases can be found in Chapters VI, VII and VIII, while a more detailed discussion is given in Chapter XI. The following data can also be found in table 5, p. 203.

Phase 0: No response to 16 hours day length and 20° C.
Phase 1: No response to 8 hours day length and 20° C.
Phase 1a: response to 8 hours day length and low temperature.
Phase 1b: no response to 8 hours day length and low temperature.
Phase 2: response to 8 hours day length and 20° C.

All phases, except phase 0, respond to 16 hours day length and 20° C. The development of the gonads proceeds from phase 0 to phase 2 (via 1a and 1b) and from there to maturity.

The last remark that must be made here concerns the action of the various external factors used in this investigation. The reader will see that in the majority of experiments day lengths of 8 or 16 hours and temperatures of 4 or 20° C have been used, and that some phases respond to these conditions while others do not. In all cases it is thought that the response would occur not only to the particular day lengths and temperatures used, but that a response would also be obtained by any day length or by any temperature above (or below) a certain threshold value, which must be somewhere between 8 and 16 hours day length and 4° and 20° C in the above examples. No experiments have been made here on the determination of threshold values, but they undoubtedly exist. This becomes clear when it is shown, for example, that 8 hours day length gives no response while 16 hours day length does. Only by assuming the existence of threshold values can the results obtained in the experiments of this investigation be compared to conditions in nature.

III. THE INFLUENCE OF TEMPERATURE ON DEVELOPMENT OF THE GONADS BETWEEN DECEMBER AND MAY

1. THE RATE OF DEVELOPMENT AT DIFFERENT TEMPERATURES

In the first two experiments animals were subjected to a certain day length combined with different temperatures. At the time of the experiments there was no opportunity to keep the temperatures constant, except that of 20° C.

Experiment 1. Adult Sticklebacks were used that had been caught in brackish water in the first week of January 1952 (gonadal phases 1b and 2). On January 8 the animals were subjected to a day length of 16 hours combined with different mean temperatures, namely 20° C (constant), 10.6° C (range 8–15°), and 9° C (range 5.4–12.5°). The number of animals in the different groups was 9, 4, and 16 respectively and all of them matured in the course of the experiment.

The animals of the 20° group matured on the average 21 days after the beginning of the experiment (range 13–29), while the corresponding figures for the 10.6° and 9° groups were 44 (28–55) and 58 (44–62) respectively. These differences are significant below the 0.05 level.

This means that the rate of development of the gonads increases with temperature. Most probably there is a temperature limit where the rate of maturation does not increase any more with the temperature and decreases with a further rise, but no data are available on this subject. Sticklebacks have been observed building nests at a temperature of 24° though it is not known whether this temperature had influenced the rate of gonadal development unfavourably.

Experiment 2. The fish had been caught in brackish water on February 10, 1953 and were subjected to experimental conditions on February 12. The gonads were in phase 1b or 2 at that time. In this case the day length was 8 hours, while the mean temperatures were 20° C (constant), 15° C (range 13–17°) and 9.1° C (range 5.5–11°).

The result was that 7 out of the 8 fish in the 20° group matured on the average 19 days after the beginning of the experiment (range 11–27), while the corresponding figures for the 15° and 9.1° groups were 22 (17–36) and 38 (28–46) respectively. In the last group five out of the seven fish matured and in the 15° group one animal out of the 6 failed to mature. The difference between the 20° and 15° groups is not significant, but that between the 20° and 9.1° and the 15° and 9.1° groups respectively is significant below the 0.05 level.

Again it is evident that also at 8 hours day length the rate of gonadal development (in phase 2) increases with temperature. The failure of some fish to mature is caused by the fact that they were in phase 1b at the beginning of the experiment, and it will be shown in Chapter VI that in this phase gonadal maturation is inhibited by 8 hours day length. In phase 2 this external condition is no longer able to prevent maturation.

In both experiments 1 and 2 the temperatures were not maintained at a constant level, except at 20°. It is possible that the fluctuations in temperature may have influenced the response of the fish and

therefore it was decided to carry out one more experiment in which the temperature was maintained at a constant level.

Experiment 3. Under-yearling fish were used that had hatched in the laboratory in April and May and were kept in the aquarium-house under almost natural conditions (see fig. 1, 1955). The average length of the animals at the beginning of the experiment on January 26 was 4.4 cm and they were presumably in phase 1b or 2 of gonadal development. The day length in all groups was 16 hours, while the temperatures were 20°, 15° and 10° C respectively. The result was that the animals in the 20° group matured on the average 32 days after the beginning of the experiment (range 20–42), while the corresponding figures for the 15° and 10° groups were 51 (36–65) and 98 (67–118) respectively. The differences between the responses in the groups are all significant below the 0.03 level. It is thus shown that the rate of maturation also increases with the temperature when the various temperatures used are maintained at a constant level.

In experiments 2 and 3 both males and females were used and there was no essential difference between the responses of the two sexes, though the number of data is too small to show whether there might be a significant difference between the duration of the response-times in the two sexes.

2. THE INFLUENCE OF LOW AND HIGH TEMPERATURES ON MATURATION AT VARIOUS TIMES BETWEEN DECEMBER AND MAY

Experiments 1 and 2 were made with fish that had been caught in their natural habitat in January and February respectively. In both cases the rate of gonadal development increased with the temperature. It was decided to do a few more experiments on this subject to see whether or not the effect of the temperature would remain the same throughout the entire period between December and May.

For this purpose animals were used that had been caught in their natural habitats and were exposed to the experimental conditions within two days of their capture. It was not possible to obtain a series of samples of fish caught at various times in one season and therefore experiments were made with samples of different years. The fish were exposed to either 8 or 16 hours day length combined with a temperature of 9° and 20° C. The data are compiled in table 1.

It can be seen from the table that when the day length is 16 hours the rate of gonadal development increases with the temperature at any time between December and May. In May, however, the difference is no longer significant. This is due to the fact that in this month the gonads

TABLE I

The influence of temperature on gonadal development in the period between December and May. Figures in brackets show range of response-times.

Date of beginning of experiment	Day length in hours	Number of fish per group		Initial phase of gonadal development	Percentage of animals responding		Average duration of response-time (days)		Difference between the duration of response-times	Level of significance of difference between the two groups
		temp. 9°	temp. 20°		9°	20°	9°	20°		
December 22 1954	16	—	12	1b and 2	—	100	—	16 (12–20)	—	—
January 8 1952	16	16	9	1b and 2	100	100	58 (44–62)	21 (13–29)	37	P < 0.01
February 12 1953	16	16	10	1b and 2	100	100	30 (21–42)	13 (8–19)	17	P < 0.01
March 10 1954	16	—	9	2	—	100	—	11 (8–14)	—	—
April 8 1954	16	5	5	2	100	100	17 (13–19)	6 (4–7)	11	P < 0.01
April 24 1954	16	5	—	2	100	—	10 (3–26)	—	—	—
May 18 1954	16	5	5	2	100	100	4 (2–9)	2 (1–4)	2	P = 0.35
December 22 1954	8	—	9	1b and 2	—	33	—	113 (35–156)	—	—
February 12 1953	8	7	8	1b and 2	72	87	38 (28–46)	19 (11–27)	19	P < 0.01
March 10 1954	8	—	9	2	—	100	—	15 (12–18)	—	—
April 8 1954	8	—	5	2	—	100	—	8 (6–10)	—	—
April 24 1954	8	5	5	2	100	100	14 (7–22)	5 (2–7)	9	P < 0.01
May 18 1954	8	5	5	2	100	100	4 (2–9)	2 (1–4)	2	P = 0,26

are already mature. The slight difference between the response-times at 9° and 20° in May must be explained by the fact that the animals are less active at the former than at the latter temperature.

However, when the day length is 8 hours, it can be seen that the rate of gonadal development increases with the temperature in only some of the animals caught between December and February. The other animals do not respond. The latter were evidently in the phase that does not respond to the stimulation of 8 hours day length (combined with either a high or a low temperature), namely phase 1b (see table 5). Animals in the latter phase do respond to the stimulus of 16 hours day length.

The data given in table 1 do not allow a comparison between the degrees of influence of the temperature in the course of the season. Information on this subject could only by obtained if the day length during the experiments was kept similar to the natural day length at the time of the year in which the experiment was made and by exposing the fish to equally large increases in temperature. But in the above cases the day lengths as well as the temperatures either increased or decreased in comparison to the natural day length and temperature, while the increment or decrement was different in the various experiments.

3. GONADAL DEVELOPMENT AND NESTBUILDING AT TEMPERATURES LOWER THAN 9° C

a. *Gonadal development*

In the previous sections it has been shown that gonadal development proceeds much more slowly at 9° than at 20° C. The question arose whether or not temperatures lower than 9° would inhibit the development of the gonads. This problem was interesting since it often happens in nature that the temperature of the water remains 4° or even lower until the middle of March.

To get information on this subject it was decided to make an experiment in which the fish were subjected to a temperature of 4° and a day length of 16 hours. The temperature of 4° was chosen since in nature fish will not often be exposed to a temperature lower than 4°; when it is freezing water of this temperature is heaviest and sinks to the bottom. The day length of 16 hours was chosen because it was known that it had a powerful stimulating effect on maturation (see Chapter IV).

Experiment 4. The animals used were adult fish that had been kept in a big basin outside the laboratory after the end of the breeding season. The experiment started on August 11, 1953. First the animals were

exposed to a day length of 8 hours and a temperature of 6°, because it was suspected that this condition might be favourable for the development of the gonads at that time of the year. This supposition later proved to be right (see Chapter VI). The fish were in phase 1a at the beginning on August 11. The above condition lasted until October 6 when the real experiment started. The animals were divided into two groups. The first group was exposed to 16 hours day length and the second group to 8 hours, while the temperature was lowered to 4° in both.

The possibility of gonadal development having taken place at 16 hours day length and 4° was tested on November 6 when the animals of both groups were subjected to 8 hours day length and 20°. The animals had partly reached phase 2 in November. In nature phase 2 is usually reached between December and May.

The result of this experiment was that the 7 animals in the first group (16 hours) matured an average of 18 days (range 11–24) after the last transfer, while only 3 out of the 5 fish of the second group (8 hours) matured. The mean date of maturation of these 3 animals was 33 days after the last transfer (range 31–35). The difference is significant at the 0.003 level. This difference between the response-times of the two groups may even have been reduced by the fact that the fish of the first group had suffered a decrease in day length from 16 to 8 hours, unlike those in the second group.

This experiment clearly shows that when the day length is long gonadal development in the phases 1b and 2 can take place at the very low temperature of 4°.

Now the fact that a long day length is able to exert a favourable effect on gonadal development when the temperature is very low is an important phenomenon. It was already mentioned that it often happens in nature that the temperature of the water remains low until the middle of March, as in 1955 and 1956. Nevertheless it was observed in these years that the Sticklebacks started to breed at the normal time of the year, namely at the middle of April. This phenomenon can be explained by the fact that the relatively long day lengths in February and March were able to stimulate gonadal development (of phases 1b and 2) notwithstanding the very low temperatures. Thus when the temperatures finally increased after the middle of March the gonads had already so far developed that only a short period was necessary to induce complete maturation (see table 1).

b. *Nestbuilding*

The foregoing experiments have shown the influence of low temperatures on gonadal development. The next question is what might be

the effect of low temperatures on animals that are already in breeding condition. Any aquarist who has kept Sticklebacks knows that the fishes show much more overt aggressive, sexual and parental behaviour when the temperature of the water is high than when it is low. A temperature of 15–20° C seems to be most favourable for the fish to show reproductive behaviour, but it is also shown at lower temperatures. The following experiments were made to determine the lowest temperature at which reproductive behaviour can still occur. The criterion for this behaviour was the nestbuilding of the male Sticklebacks.

Experiment 5. The animals used were adult fish that had built nests in the beginning of May, 1954, at a temperature of 16° C and a natural day length. On May 8 the animals were exposed to a day length of 16 hours combined with three different temperatures, namely 8, 6, and 5° C. In the first group 8 animals were used and all built nests, on the average 11 days after the transfer (range 4–16). All three animals kept at 6° responded and their average nestbuilding date was 12 days after the transfer (range 10–13). But only one out of the three animals in the 5° group built a nest (15 days after the transfer), while another animal started to collect some material but stopped this activity after a couple of days.

The conclusion that can be drawn from this experiment is that 5° is the minimum temperature at which nestbuilding can still occur. No observations were made on the occurrence of other reproductive activities, but it is known that all these activities are shown less frequently at temperatures below 10°.

However, in the above cases where nestbuilding occurred at low temperatures it was observed that all nests showed some deficiencies when compared to nests built at higher temperatures. The most remarkable difference was that the nests built at low temperatures showed a loose structure as if the material had not been glued together very well. Normal nests are tightly glued together. It is probable that the secretion of glue by the kidneys was impaired by the sudden transfer to the low temperature.

A second point of difference was the fact that at high temperatures the nests are usually built within 24 hours or less. But at the low temperatures it took the fish more than three days to finish the nest. In some cases it even lasted longer and one fish (in the 5° group) never finished its nest though it had started building.

The reader may have observed that the response-times of the fish with respect to nestbuilding after the transfer were rather long, namely 11, 12 and 15 days respectively. In table 1 it was shown that in May usually only 2–4 days pass between these two dates. It is supposed that the long interval was due to the fact that the animals were rather suddenly (within 2 days) exposed to a temperature drop of more than 8°.

In the above experiment animals were used that had already attained breeding condition before they were exposed to the low temperatures. Moreover, they had been exposed to a sudden decrease in temperature which might have effected their general condition unfavourably. Therefore, another experiment was designed in which the animals had been kept at a low temperature from the beginning of their gonadal development.

Experiment 6. In this case animals were used that had finished their reproductive period under 8 hours day length and 20° C. They were kept under the same condition for about one month, when they were exposed to a temperature of 4°, while the day length remained 8 hours. In Chapters VI and VII it will be shown that this condition is favourable for gonadal development of phase 1a. When they were subsequently exposed to a day length of 16 hours and a slowly increasing temperature 3 out of the 6 animals used built a nest when the temperature had increased to 6°. The remaining animals built nests at about 9°. Thus in this case nestbuilding began at a temperature of 6° (in the foregoing experiment at 5°). In the present experiment the nests had a quite normal appearance, i.e. they were tightly glued together. This is in contrast with the nests described in experiment 5. This fact supports the idea that in experiment 5 the secretion of glue by the kidneys had been impaired by the sudden decrease in temperature to which the animals had been exposed.

4. DISCUSSION

The experiments described in this chapter have shown that when the day length is 16 hours the rate of gonadal development increases with the temperature at any time between December and May. In case the day length is 8 hours the above acceleration is only shown by some of the animals caught in December, January and February. This is due to the fact that the gonadal condition of the fish that did not respond was such that they were unable to react to the stimulus of 8 hours day length together with a low or a high temperature (phase 1b). We shall return to this subject later in Chapters VI and VII.

Courrier (1922) and Craig-Bennett (1931) working with the same species also showed that a high temperature accelerated maturation in the early months of the year. Merriman and Schedl (1941) observed that in the 4-spined Stickleback (*Apeltes quadracus*) a high temperature accelerated spermiogenesis and also the development of the first stages of oogenesis. However, the later stages of oogenesis did not respond

to a high temperature. It seems that in this species temperature affects the sexes differently, unlike the 3-spined Stickleback, where there does not seem to be any essential difference. BURGER (1939 a, b) and MATTHEWS (1939b) working with the Killifish (*Fundulus heteroclitus*) and MEDLEN (1951) with *Gambusia affinis* showed that in both species spermiogenesis is regulated by the temperature, the process being speeded up by a high temperature.

BULLOUGH (1939) found that in the Minnow (*Phoxinus laevis*) a high temperature in mid-winter had no influence on gonadal development. A long day length could induce maturation at that time, but only when the temperature exceeded 10° C. Thus, temperature still plays a rôle in this species.

VERHOEVEN and VAN OORDT (1955) showed that in the Bitterling (*Rhodeus amarus*) the male could be induced to mature in mid-winter by exposure to a high temperature, irrespective of a long or short day length. But in the female precocious oviposition could only be induced by a high temperature combined with a long day length.

The investigations mentioned above were made with animals that have their breeding season in spring or summer and it is therefore conceivable that they show a positive response to a high temperature. But what will be the response to temperature of animals that have their breeding season in autumn and winter? Unfortunately there seem to be very few data available on this subject. FABRICIUS (pers. comm.) was able to accelerate spawning in the char (*Salmo alpinus*) by lowering the temperature of the water in autumn following exposure to relatively high temperatures in spring and early summer. KAZANSKI (1953), cited by PICKFORD and PETRUNKEVITCH, (1955) observed that the autumn race of the Caspian Sturgeon (*Acipenser güldenstädti persicus*) spawns when the temperature falls to 16° or 17° C.

From the examples it is clear that, although most species respond to changes in the temperature of the water, they each seem to have developed in their own way a response-mechanism to that change, which times the onset of their breeding season in a way which is most favourable for the survival of the species. Thus, there is much variation with respect to the influence of the temperature on gonadal development between the different species and sometimes even between the two sexes of one species.

The scope of the present study does not allow a review of the experiments made on the influence of temperature on gonadal development in other species of Vertebrates. But it can be said that generally temperature is much more important in cold than in warm blooded animals. For literature the reader is referred to "The Memoirs of the Society for Endocrinology" no. 4, 1955.

IV. THE INFLUENCE OF DAY LENGTH ON DEVELOPMENT OF THE GONADS BETWEEN DECEMBER AND MAY

1. THE RATE OF DEVELOPMENT AT DIFFERENT DAY LENGTHS

Experiment 7. The animals used were under-yearling fish hatched in the laboratory in April and May, 1953 and reared in the aquarium-house where the conditions were almost similar to those in nature (see fig. 1). The experiments started on January 6, 1954, when the mean length of the fish was 5.1 cm. At this time their gonads were in phase 2. The animals were exposed to a temperature of 20° C, while the day length was either 8 or 16 hours. Both males and females were used and all animals in both groups matured.

The average date of maturity of the 6 fish in the 16-hour group was 49 days after the beginning of the experiment (range 29–63), while the corresponding figure for the 6 fish in the 8-hour group was 122 days (range 88–168). This difference is significant at the 0.002 level. There did not seem to be an essential difference between the responses of males and females, though the number of data was too small to show a possible difference in the duration of the response-times of the two sexes.

The conclusion is that the rate of gonadal development increases with the day length.

Since the above experiment was made with under-yearling fish it was decided to carry out an experiment also with adult fish.

Experiment 8. The fish were caught in the sea on December 15, 1954 and were afterwards kept in the aquarium-house until December 22 (for conditions see fig. 1). On this date they were transferred to a temperature of 20° and a day length of either 8 or 16 hours. Their gonads were in phase 1b or 2 at the beginning of the experiment. The results are given in figure 2 which will be fully discussed later with respect to the other data (Chapter V).

The 12 animals in the 16-hour group matured on the average 16 days after the transfer (range 12–20). But of the 9 fish that were used in the 8-hour group only 3 matured. The average response-time of these 3 animals was 113 days (35, 149 and 156 respectively). The differences between the animals that matured in the two groups is significant at the 0.007 level. The failure of the remaining animals to respond must be explained by the fact that at the beginning of the experiment their gonads had evidently been in phase 1 (see table 5).

The main conclusion of these experiments is that gonadal development is accelerated by a long day length in adult as well as under-yearling fish.

In the two foregoing experiments the influence of the day length was determined at a temperature of 20° C. It was decided to make an experiment at a lower temperature also in order to determine whether or not the effect of day length would remain the same.

Experiment 9. The adult fish that were used had been caught in brackish water on February 10, 1953. On February 12 they were subjected to a mean temperature of 9° C (range 7–13°), while the day length was either 8 or 16 hours. At this time their gonads were in phase 1b or 2.

The result was that all 10 fish in the 16-hour group matured. The average response-time was 30 days after the beginning of the experiment (range 21–42). The corresponding figure for the 5 animals (out of the 7) which matured in the 8-hour group was 38 days (range 28–46). The difference between the groups is significant at the 0.025 level.

The conclusion from this experiment is that a long day length is also able to accelerate maturation at a low temperature. A similar effect has already been shown in experiment 4 in which gonadal development took place at a temperature of 4° under the influence of a day length of 16 hours.

The conclusion of the experiments described in this section is that a long day length accelerates gonadal development, both at low and high temperatures. There does not seem to be an essential difference between the responses of males and females.

2. THE INFLUENCE OF SHORT AND LONG DAY LENGTHS ON MATURATION AT VARIOUS TIMES BETWEEN DECEMBER AND MAY

The following experiments were made in order to determine the influence of day length at two different temperatures in the course of the season between December and May. A similar set of experiments on the influence of temperature has already been given in table 1.

Animals were used that had been caught in their natural habitats and had been exposed to the experimental conditions shortly after arrival in the laboratory. The fish were exposed to a temperature of either 9° or 20° C, combined with a day length of 8 and 16 hours. The data are compiled in table 2.

It is clear that at any time between December and April the rate of gonadal development increases with the day length, both at a low and a high temperature. Later the differences are no longer significant. This result means that all stages of gonadal development attained at

TABLE 2

The influence of day length on gonadal development in the period between December and May. Figures in brackets show the range of response-times.

Date of beginning of experiment	*Temp. in °C.*	*Number of fish per group*		*Initial phase of gonadal development*	*Percentage of animals responding*		*Average duration of response-time (days)*		*Difference between the duration of the response-times*	*Level of significance of difference between the two groups*
		day length 8h.	*day length 16 h.*		*8 h.*	*16 h.*	*8 h.*	*16 h.*		
December 22 1954	20	9	12	1b and 2	33	100	113 (35–156)	16 (12–20)	97	P < 0.01
January 8 1952	20	—	9	1b and 2	—	100	—	21 (13–29)	—	—
February 12 1953	20	8	10	1b and 2	87	100	19 (11–27)	13 (8–19)	6	P = 0.056
March 10 1954	20	9	9	2	100	100	15 (12–18)	11 (8–14)	4	P < 0.01
April 8 1954	20	5	5	2	100	100	8 (6–10)	6 (4–7)	2	P < 0.01
April 24 1954	20	5	—	2	100	—	5 (2–7)	—	—	—
May 18 1954	20	5	5	2	100	100	2 (1–4)	2 (1–4)	0	
January 8 1952	9	—	16	1b and 2	—	100	—	58 (44–62)	—	—
February 12 1953	9	7	10	1b and 2	72	100	38 (28–46)	30 (21–42)	8	P < 0.03
April 8 1954	9	—	5	2	—	100	—	17 (13–19)	—	—
April 24 1954	9	5	5	2	100	100	14 (7–22)	10 (3–26)	4	P = 0.42
May 18 1954	9	5	5	2	100	100	4 (2–9)	4 (2–9)	0	

this time of the year (from phase 1b to maturity) are influenced favourably by long day lengths.

On page 123 it was mentioned why the data of table 1 could not be used for a comparison between the degree of influence of temperature in the course of the season. On similar grounds, but with respect to the day length, it is clear that the data of table 2 cannot be compared with regard to the degree of influence of day length in the course of the season. It is for the same reasons impossible to compare the influence of day length and of temperature in the course of the season.

3. THE INFLUENCE OF DIFFERENT INTENSITIES OF LIGHT ON GONADAL DEVELOPMENT

In Chapter II it was mentioned that most of the experiments described in this paper were carried out in temperature rooms where all tanks received a light intensity of 25–30 f.c. But in many cases the results achieved with these light intensities were compared with observations in nature where usually the intensities are much higher. Therefore, it was necessary to know whether light of higher intensities than 25–30 f.c. would give different results.

a. *The influence of light of high intensity*

Experiment 10. The light intensity that was used here was about 300 f.c. This light intensity can be roughly compared with that of a cloudy summer day at the middle of the morning or afternoon.

The sources of light used were Philips fluorescent tubes of 40 Watt, 2400 lumen. Four tubes were suspended above two aquaria about 30 cm above the water-level. Though the tubes did not produce much heat, the temperature of the water nevertheless fluctuated daily between 20° and 22.5° C. The temperature of the water of the control fish was made to follow the same fluctuation by means of a heater that was switched off during the night.

The control fish received light of 25–30 f.c., but unfortunately it was impossible to use the same tubes as were used for the experimental fish. The former were illuminated with ordinary electric bulbs. But it will be shown in the next section that this difference in the quality of the light cannot have influenced the results.

The animals used were under-yearling fish that hatched in April and May, 1955 and had been kept in the aquarium-house until July 11. On that date they were transferred to a day length of 8 hours and a temperature of 20° C. In Chapter VI it will be shown that this condition inhibits maturation in animals that are in phase 1 of gonadal development. The real experiment started on December 30 when

some of the fish were subjected to light of high intensity. In both groups the day length remained 8 hours while the temperature varied between 20 and 22.5 degrees.

The result of this experiment was that no experimental or control fish matured. The animals were kept under the above conditions for 52 days. Thus it is clear that the increase in the intensity of the light did not induce maturation under these conditions and also that light of a high intensity (± 300 f.c.) was no more effective than light of an intensity of 25–30 f.c.

On February 20 the second part of the experiment started. Now both groups received a day length of 16 hours, while the other conditions remained the same.

The result was that all control fish (9) matured on the average after 35 days (range 28–43), while the corresponding figure for the 5 experimental animals was 40 (range 31–50). This difference is not significant.

The conclusion from this experiment is that light of a high intensity (± 300 f.c.) is no more effective in inducing maturation than light of a much lower intensity (25–30 f.c.). Therefore, the results obtained with light intensities of 25–30 f.c. may be compared with observations in nature.

b. *The influence of light of low intensity*

To see whether or not light of a low intensity would affect gonadal development it was decided to carry out an experiment on this subject also.

Experiment 11. Both experimental and control groups were kept in a room where the light intensity that fell on the aquaria was 25–30 f.c. (light from ordinary bulbs). However, in the experimental group the light intensity was reduced by covering all 4 sides of the aquarium with hard-board plates, while the top was screened with a double layer of white netting with a mesh of 1 mm diameter. In this way the light intensity on the surface of the water was reduced to about 15 f.c. The difference in light intensity to which the fish were exposed, however, was still greater, for in the control group the sides of the aquarium were not covered with hard-board plates.

Under-yearling fish were used that were about 8–10 months old when the experiment started on December 17. The gonads were in phase 1b at the beginning of the experiment. Both groups received a day length of 16 hours and a temperature of 20° C.

The result was that the 6 animals in the control group matured on the average 28 days after the beginning of the experiment (range

25–32), while the 6 fish in the experimental group matured on the average 32 days after the beginning (range 29–35).

The difference between the response-times in the two groups is significant at the 0.04 level. This indicates that a light intensity of about 15 f.c. may have a retarding effect on gonadal maturation compared to a light intensity of 25–30 f.c. It seems possible that the rate of gonadal development will decrease still more with a further decrease in the intensity of the light.

4. THE INFLUENCE OF LIGHT WITH DIFFERENT SPECTRA ON GONADAL DEVELOPMENT

The experiments mentioned here were not made primarily to study the influence of the quality of light on gonadal development. The reason why they are described is that some of the previous experiments had been made with light from different sources, such as ordinary bulbs, fluorescent tubes and day light. In order to be justified in making comparisons between the experiments with the different light sources, it was necessary to know the influence of the light-quality.

In Chapter II it was mentioned that in the majority of experiments ordinary electric bulbs of 75 and 150 Watt were used. Such bulbs have a continuous spectrum with a large part of their energy in the infra-red and with gradually less energy from red to blue.

Other sources of light that have been used were fluorescent tubes of 25 and 40 Watt (Philips). Four different types of tubes were used.

1. "Wit": energy peak around 5800 Å, with a relatively high proportion of energy between 4500 and 5800 Å.
2. "Wit de Luxe": high peak of energy around 6500 Å and a lower one around 5800; again a relatively high proportion of energy between 4500 and 5800 Å, though less than in 1.
3. "Warmtint": peak around 6000 Å and a rather high proportion of energy between 5500 and 6500 Å.
4. "Daglicht": a high proportion of energy between 4500 and 6500 Å. Of these four types the last one has the highest proportion of blue light. None of the tubes, however, has much energy in the infra-red, unlike the ordinary bulbs.

Experiment 12. In this case the effect of the above four types of tubes on gonadal development was studied. The experiment was made with fish caught in their natural habitat on March 1. They were exposed to a day length of 8 hours and a temperature of 20° C. The day length of 8 hours was chosen because at this time of the year a longer day length results in a maturation of the gonads within a very short time, so that possible differences between the various groups would have become too small. The gonads were in phase 2. The results of this experiment were as follows:

a. the group under tube 1: 3 animals; mean response time 17 days; range 5–23 days
b. the group under tube 2: 5 animals; mean response time 17 days; range 11–22 days
c. the group under tube 3: 5 animals; mean response time 15 days; range 8–23 days
d. the group under tube 4: 6 animals; mean response time 16 days; range 11–27 days

This result shows that there is not much difference between the effect of light with different spectra, while the differences proved to be not significant.

Experiment 13. In this case a group of animals was exposed to light coming from each of the four tubes described above, but the results obtained in the four groups were added, because the previous experiment had shown that there is no difference between the effect of the tubes. This combined result was compared with that obtained with a group of fish subjected to light coming from ordinary bulbs. The fish came from the same stock as those of the previous experiment. On March 1 they were subjected to a day length of 8 hours, while the temperature was on the average 12° C (increasing from 8–17°).

The result was that the animals receiving light from bulbs matured on the average 17 days after the beginning of the experiment (range 9-24). The combined data of the four tubes showed that the mean date of maturation was 19 days after the beginning of the experiment (range 10–24). The result with tube 1 in this experiment was that maturation took place on the average 17 days after the beginning of the experiment (range 9–24). This was the type of tube that was used in experiment 10.

The result of the present experiment shows that there is not much difference between the effects of light from tubes and bulbs, while the differences proved to be not significant.

Experiment 14. Here the effect of light coming from tubes was compared with that of natural day light falling through the thick glass roof of the aquarium-house. The experiment started on February 12 with animals caught in their natural habitat two days before. The fish were in phase 1b or 2 of gonadal development. The temperature was 20° C in both groups and the day length 8 hours. In the aquarium-house the latter was achieved by covering the aquarium at 5.00 p.m. and uncovering it at 9.00 a.m.

The summarized result of the experiments with the tubes was that maturation took place on the average 19 days after the beginning of the experiment (range 15–40). The animals receiving "natural" day light in the aquarium-house matured also on the average 19 days after the beginning (range 11–27).

The conclusion is that there is not much difference between the effects of light from tubes and light (almost) similar to that of normal day light; the difference is not significant. The fact that the intensities of the light differed considerably (50 f.c. in the experiments with the tubes and about 200 f.c. in the aquarium-house) cannot have influenced the results of this experiment because it is known that light intensities above 25–30 f.c. are not able to accelerate maturation more than intensities of 25–30 f.c. (experiment 10).

No experiments were made to study the effect on gonadal development of natural day light compared with that of bulbs, but on the grounds of the results of the previous experiments it can be concluded that there will be no difference between the effects of these two sources of light. Therefore, it is justified to compare experiments made with natural day light and light coming from tubes and bulbs as long as the light intensity that is used is higher than 25 f.c. The experiments were rather crude but still sufficiently accurate for their purpose.

5. DISCUSSION

The experiments described in this Chapter have conclusively shown that in the period between December and May (gonadal phases 1b and 2) maturation of the gonads is accelerated by long day lengths, both when the temperature is low and high.

Van den Eeckhoudt (1946) and N. Tinbergen (pers. comm.) also

found that a long day length had a favourable influence on gonadal development in the 3-spined Stickleback. However, these results are not in agreement with those of CRAIG-BENNETT (1931), who found that in the Stickleback the length of day did not affect the development of the gonads. This author based his conclusions on the criterion whether or not the day length affected the development of the secondary sex characters; their development is known to depend on the action of gonadal hormones. But in Chapter VIII of the present investigation it will be shown that in all cases in which artificial stimulation results in a very rapid maturation of the gonads, the development of the secondary sex characters lags far behind. This is one of the reasons that CRAIG-BENNETT was unable to detect any influence of long day lengths in the early months of the year.

In the second place CRAIG-BENNETT used light with an intensity of about 4 f.c. It was shown in section 3 of the present chapter that the response-time to light of about 15 f.c. was significantly longer than that of an intensity of 25–30 f.c. Therefore, in the case of GRAIG-BENNETT's experiments the intensity of 4 f.c. might have been too weak a stimulus to cause an observable effect.

Also the fact that CRAIG-BENNETT used day lengths equal to the natural day lengths in January and February plus one hour and a half of artificial illumination may have been partly responsible for his failure to detect any effect of the day length, because it was relatively short and thus may not have had much effect. These three factors were the main reasons for the undoubtedly wrong conclusion of CRAIG-BENNETT with regard to the influence of day length on the development of the gonads.

MERRIMAN and SCHEDL (1941) showed that in the male 4-spined Stickleback (*Apeltes quadracus*) the day length does not influence spermiogenesis in winter, but that in the female complete oogenesis can only be induced by exposing them to long daily photo-periods. BURGER (1939 a,b) and MATTHEWS (1939b) working with *Fundulus heteroclitus* and MEDLEN (1951) with *Gambusia affinis* were unable to demonstrate any influence of light on maturation. BULLOUGH (1939) found that in the Minnow (*Phoxinus laevis*) a long day length could induce maturation, but only if the temperature exceeded 10° C. VERHOEVEN and VAN OORDT (1955) working with the Bitterling (*Rhodeus amarus*) showed that in the male the day length did not affect maturation, but that in the female a long day length was effective when combined with a high temperature. HARRINGTON (1950) found that in *Notropus bifrenatus* a precocious maturation could be induced by a long day length and HAZARD and EDDY (cited by HOAR, 1955) reported a similar result in another species.

In fish spawning in autumn day length also plays an important rôle, though in this case it is a short or decreasing day length which is effective. HOOVER and HUBBARD (1937) induced maturation in the speckled trout (*Salvelinus fontinalis*) considerably in advance of the normal breeding season by subjecting them to increasing day lengths early in the year and to subsequent decreasing day lengths in the summer. No other investigations on this subject are known in fish, but in mammals BISSONNETTE (1941) and YEATES (1947) have found that in goats and sheep precocious breeding can be induced by short day lengths.

No data on the influence of the intensity and quality of light are available in fish, but in birds and mammals it is known that light of low intensity greatly retards maturation (see BISSONNETTE, 1938, and review of studies on this subject in birds by BENOIT and ASSENMACHER, 1953b). In birds it has been shown that the wave-length of light also plays a rôle in gonadal development (BENOIT and ASSENMACHER, 1953b).

Surveying the results of experiments on photoperiodicity in other Vertebrates it can be said that generally the day length seems to be more important in warm than in cold blooded animals. With regard to the influence of the temperature the reverse seems to be true. Again it is clear that, though the length of day plays an important rôle in many vertebrate species, each species has developed in its own way a response-mechanism to this external factor and the result is that breeding takes place in that part of the year which is most favourable for the rearing of young.

V. THE INFLUENCE OF GRADUALLY INCREASING DAY LENGTHS AND TEMPERATURES

In the foregoing chapters it has been shown that gonadal development in fishes responded very well to a sudden increase in day length from 8 to 16 hours and to a sudden increase in temperature of 10 or more degrees. However, it is still a wide-spread idea that the *gradual increase* in day length and temperature *as such*, to which the animals in their natural habitats are exposed early in the year, would be responsible for the development of the gonads. For autumn breeders the gradual decrease in day length and temperature would be effective. In order to obtain more information on the rôle of increasing day lengths and temperatures on maturation in the Stickleback the following experiment was designed.

Experiment 15. The fish that were used (only males) had been caught in the sea on December 16, 1954 (temperature about 6° C). After

transportation to the laboratory they were kept outside the building where the day length and temperature conditions were natural. On December 22, the shortest day of the year (about 8 hours), they were exposed to 5 different external conditions. At this time some of the animals were in phase 1b while others were already in phase 2.

Group I: day length 16 hours; temperature 20° C.

Group II: artificial increase in day length (15 min. per 2 days) from 8–16 hours; temperature 20° C.

Group III: day length 8 hours; artificial increase in temperature (0.5 degree per day) from 6–20° C.

Group IV: day length 8 hours; temperature 20° C.

Group V: this group was kept in the aquarium-house where the day length conditions were similar to those in nature; the temperature followed the same gradual increase as in nature but was usually higher (fig. 1).

The result of this experiment is given in fig. 2.

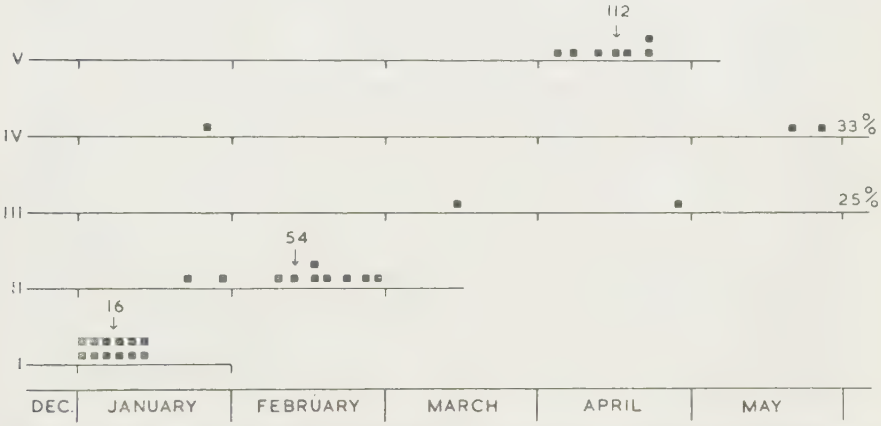

Fig. 2. Experiment 15. Dates of attainment of maturity of 5 groups of fish exposed on December 22 to various external conditions. I. Day length 16 hours; temperature 20° C. II. Increasing day length from 8–16 hours; temperature 20° C. III. Day length 8 hours; temperature increasing from 6–20° C. IV. Day length 8 hours; temperature 20° C. V. Natural day length and temperature conditions. The arrows and accompanying figures indicate the average date of maturity and the mean duration of the response-time of the groups. The percentages indicate how many of the fish used matured in the various groups. Where no percentages are given all fish attained maturity.

The animals from the first group (16 hours; 20°) matured first of all, on the average 16 days after the beginning of the experiment (range 12–20). The animals of the second group (increasing day length; temperature 20°) only matured on the average 54 days after the be-

ginning (range 25–68). This difference is significant at the 0.01 level. Thus fishes exposed to a sudden long day length respond much earlier than those receiving a gradual increase in day length.

Of the 9 animals in the fourth group (day length 8 hours; temperature 20°) only 3 matured in the course of the experiment. Of the third group (day length 8 hours; increasing temperature) only 2 out of the 8 animals matured. The difference between these two groups is not significant. This means that at 8 hours day length it makes no difference whether the fish are exposed to a sudden increase in temperature or to a gradual increase in temperature. In both cases gonadal maturation proceeds at the same slow speed, or does not take place.

However, there is a significant difference (P smaller than 0.01) between the results of groups III and IV and those of groups I and II. This is caused by the fact that at this time of the year a long day length together with a high temperature is evidently much more stimulating than a high temperature alone (see also tables 1 and 2). The failure of some of the animals to respond (groups III and IV) must be explained by assuming that at the beginning of the experiment they had been in a phase of gonadal development that cannot be stimulated by a day length of 8 hours (see Chapters VI and VII).

Finally the result of group V shows that in case both the temperature and the day length increase gradually, the effect of this treatment is much weaker than that obtained in group I. The animals in the former matured on the average 112 days after the beginning of the experiment (range 103–119). The difference between this response and that of group I (and also of group II) is significant at the 0.001 level. There is no significant difference between the duration of the response-times in group V and that of the animals which responded in groups III and IV. However, in group V all animals that were used attained maturity, while in the latter groups only 25% and 33% respectively matured. This shows that in at least some of the animals the external conditions of group V were better than those in groups III and IV. This difference is caused by the fact that the day length in group V gradually became longer than 8 hours.

The conclusion from this experiment is that the development of the gonads is much more accelerated by a sudden increase in day length and temperature than by a gradual increase in each of these components, or of both at the same time. This means that the total amount of light and of temperature received is more important than the gradual increase as such.

It must be emphasized that in the above experiment the effect of the gradual increase in day length and temperature as such was studied

with respect to the *rate of development* of the gonads at the particular time of the year. However, it is possible that the increase as such might still have a favourable effect on another process, namely on the *initiation of development* of a certain gonadal phase. Before this point will be discussed it is necessary to explain first what is meant by initiation.

In Chapter VI it will be shown that the development of one of the gonadal phases cannot take place under a certain external condition. This means that in the absence of this external condition the development of the particular phase comes to a standstill. But as soon as the external stimulus is present the development takes place. In this case it is said that the external stimulus initiated the development. Once the process has been initiated it is possible that external stimuli which were ineffective before the initiation are then able to influence the rate of development of this phase. A similar influence can also be exerted by the external stimulus which initiated the process.

Now in the above experiment it was shown that the gradual increase as such of day length and temperature has no important influence on the rate of gonadal development. However, it may still have an initiating effect on a certain process, though conclusions on this subject cannot be drawn from this experiment.

The influence on initiation must be studied in a different way. With respect to light this has been done in birds by Burger (1939c). This investigator showed that in the Starling a constant day length of 10¼ hours was unable to induce maturation. But a gradual increase in the day length from 9–9¾ hours over a 16-day period resulted in the initiation of spermiogenesis. He could also show that the increase was only effective above a certain threshold value of the duration of the daily photo-period. It would certainly be worth-while to make a similar experiment with 3-spined Sticklebacks. However, if it were true that the gradual increase in day length has an initiating influence, it certainly would not be the only initiating factor, for it will be shown in Chapter X that animals kept under constant external conditions of 16 hours day length and 20° C show alternative reproductive and non-reproductive periods.

A number of investigators working with birds have already considered the gonadal cycle in relation to the total amount of light (in hours) or to the summation of day lengths rather than to the increase in day length as such (Burger, 1939 c,d; Wolfson, 1952; Bartholomew, 1949, cited by Wolfson, 1952). They also pointed out that threshold values of the daily photo-period exist, below which total light is not effective. In the Stickleback such a threshold value for stimulation by day length exists, for in Chapter VI it will be shown that under 8

hours day length and 20° C no maturation occurs (when the fish are in a certain phase of gonadal development), while they do respond to 16 hours day length and 20° C in the same phase. However, I do not yet know where the threshold value for photostimulation lies; it must be somewhere between 8 and 16 hours.

With regard to the effect of summation of the daily temperature on gonadal development little seems to be known. KLUYVER (1951, 1952) studying the dates of egg-laying of the Great Tit in nature obtained data which indicated that the heat-budget from the middle of March onward is an important factor determining the start of egg-laying.

VI. CRITICAL EXTERNAL FACTORS IN THE PROCESS OF MATURATION

The term critical factor is used to indicate a factor without which a certain process cannot take place. By maturity is meant the phase of gonadal development in which ripe sperm and ova are present and whereby the animals show full reproductive behaviour.

The animals used in the experiments were either under-yearling fish about 5 months old or adult fish that were in the first month of their post-breeding condition. In both cases it was assumed that the gonads had not yet started to develop or had only slightly developed at the beginning of the experiments.

1. INHIBITION OF MATURATION UNDER 8 HOURS DAY LENGTH AND 20° C

Experiment 16. The animals used were under-yearling fish that had hatched in April and May, 1953, and had been reared in the aquarium-house (conditions see fig. 1). On September 29, when they had a mean length of 4.1 cm, the fish were subjected to a temperature of 20° C and either 8 or 16 hours of daily illumination. Both males and females were used and all animals were in phase 1 at the beginning of the experiment.

The 11 animals in the 16-hour group matured on the average 47 days after the beginning of the experiment (range 38–70). The fish then were 8 months old and had a mean length of 4.7 cm. None of the 10 animals in the 8-hour group had responded 6 months after the beginning of the experiment (mean length 5.2 cm). But when at that time they were transferred to 16 hours day length (the temperature remained 20° C) all 7 animals matured. The mean response-time was 31 days after the transfer (range 20–37). A similar result was obtained

when 3 animals of the 8-hour group were transferred to 16 hours day length about 3 months after the beginning of the experiment. The animals then matured 14, 25 and 47 days respectively after the transfer (average 29 days).

The results of this experiment show that maturation in under-yearling fish about 5 months old can be prevented by subjecting them to 8 hours day light and 20° C. Since fish of the same age that had been exposed to 16 hours day length and 20° attained maturity, it is evident that in this case 8 hours day length, or the combination of 8 hours day length with a temperature of 20°, had been an inhibiting factor in the process of maturation. The differences between the responses of males and females are not significant.

Experiment 17. This experiment is identical to no. 16 and was made to confirm the results obtained in the latter experiment. It was carried out in 1954 with under-yearling fish (hatched in April and May) that had been reared in the aquarium-house (conditions see figure 1.) The experiment started on October 9 when the fish had a mean length of 3.9 cm. They were exposed to a temperature of 20° C and a day length of either 8 or 16 hours. Both males and females were used and all fish were in phase 1.

The results of this experiment were similar to that of the previous one. All 6 fishes in the 16-hour group matured; average response-time 41 days (range 31–54). However, in the 8-hour group none of the animals attained maturity within 10 months, when the experiment was broken off. Three months after the beginning of the experiment a group of 4 animals was transferred to a day length of 16 hours, while the temperature remained 20°. All of these animals responded; average response-time 28 days (range 19–37).

The conclusion from this experiment is identical to that of the previous one. The short day length, either combined with 20° C or not, was an inhibiting factor in the process of maturation. There was no significant difference between the responses of males and females.

The foregoing two experiments were made with under-yearling animals and it was decided to repeat the experiments with adult fishes, since it might be possible that the reaction of young fish would be different from that of adults.

Experiment 18. The animals used were adult males that had built nests under experimental conditions between February and April (1954) and had subsequently been kept in a basin outside the laboratory under natural conditions (see fig. 1). On October 9 they were exposed to a temperature of 20° C and a day length of either 8 or 16 hours.

The result was that the 10 animals in the 16-hour group matured on the average 21 days after the beginning of the experiment (range 12–35). But none of the animals in the 8-hour group responded in the course of 9 months, when the experiment was broken off. At this time only 2 out of the original number of 10 fish in this group were still alive.

The conclusion is that also in adult fish in which the gonads are not yet developed or only in the very first stages of development 8 hours day length, whether combined with a temperature of 20° or not, is an inhibiting factor in the process of maturation.

A similar result was obtained in experiment 19. Also in this case 8 hours day length and 20° prevented maturation in adult and young animals even when they were maintained for over one year under the same external condition.

From the above experiments three conclusions can be drawn. In the first place it has been shown that under the condition of 8 hours day length and 20° C maturation can be prevented in animals that are in an early phase of gonadal development at the beginning of the experiment. The latter must be emphasized, since it has been shown in previous experiments (see table 1 and 2, and experiment 7) that with animals in a later phase of gonadal development at the beginning of the experiment, the above condition is no longer able to prevent maturation. This means that there must be a certain phase (phase 1) in the development of the gonads at which further development comes to a standstill when the day length remains 8 hours and the temperature 20° C. This point will be discussed in some detail later.

In the second place the fact that animals maintained for a long time at 8 hours day length and 20° do not mature, but attain maturity as soon as they are transferred to 16 hours day length and 20°, means that the latter condition is a critical factor in the process of gonadal maturation. It cannot be concluded yet whether it is the long day length, or the combination of the long day length with the high temperature, which is responsible for maturation.

In the third place the results obtained with the under-yearling animals show that, though complete maturation is prevented, some development of the gonads can still take place under 8 hours day length and 20°. This is shown when the duration of the response-times to 16 hours day length and 20° are compared. In experiment 16 the animals responded after 47 days when exposed to this condition on September 29. Three months later, when they were transferred from 8 to 16 hours, they responded after 29 days, while again three months later it took them 31 days after the transfer. In experiment 17 the corresponding figures were 41 and 28 days (no transfer took place after 6 months). In the former experiment the difference between

47 and 29 days is not significant (because of the great scatter in the data), but that between 47 and 31 days is significant at the 0.001 level. This means that during exposure to 8 hours day length and 20° the gonads of the animals in experiment 16 showed a certain amount of development, though they did not mature. The difference between 41 and 28 days (experiment 17) is almost significant ($P = 0.067$) and therefore also in this case the gonads showed a tendency to develop a little under 8 hours day length and 20°, though they did not mature.

A similar development up to a certain stage may also take place in the gonads of adult fish though no data on this subject are available.

2. LEVEL OF GONADAL DEVELOPMENT OF FISH MAINTAINED FOR LONG PERIODS OF TIME AT 8 HOURS DAY LENGTH AND 20° C

In the previous section it has been shown that in animals exposed to 8 hours day length and 20° C at a very early phase of gonadal development some development may take place though the animals do not mature. It has also been mentioned that the standstill most likely occurs at a definite phase of development (phase 1). Now the question arises whether the gonads remain in this particular phase of development during exposure to the above condition no matter how long it lasts, or whether the gonads may show fluctuations in development, i.e. fluctuations between total regression and phase 1.

It seems that such a question can only be answered histologically, but the results obtained with young animals in experiments 16 and 17 led to an attempt to answer this question in another way.

In experiments 16 and 17 it was shown that when under-yearling fish are exposed to 16 hours day length and 20° C in September and October respectively, thus in a very early phase of gonadal development, it takes them longer to respond to this condition than when they have first stayed for 3 or 6 months under 8 hours day length and 20°. Now the duration of the response-time to 16 hours day length and 20° may give information on the stage of gonadal development of animals kept for various periods of time under 8 hours day length and 20°.

Experiment 19. To obtain data on this subject animals were kept for various periods of time under 8 hours day length and 20° C and were then transferred to 16 hours day length, while the temperature remained 20° C. The animals used were divided into two groups. One group consisted of fish that had spawned at least one time in their life before the experiment started; these were named the

adults. The other group consisted of fish that had never spawned before and were named young fish. All animals in both groups were at a very early phase of gonadal development (phase 1) at the beginning of the experiment in order to be sure that no maturation would take place at 8 hours day length and 20° C. The results are given in fig. 3.

It follows from the data that the adult fish mature on the average 20.3 days after the transfer (range 14–27). In the young the average duration of the response-time is 26.5 days (range 15–46). This difference between the response-times of the adults and young is significant (P between 0.005 and 0.008). This means that it takes fish that have never spawned before a longer time to attain maturity than animals that have spawned at least once.

Another conclusion that can be drawn from the data is that it always takes the animals at least 14 days to respond to the stimulus of 16 hours day length and 20°, no matter how long they have been kept under 8 hours day length and 20°. This means that the phase at which gonadal development comes to a standstill under 8 hours day length and 20° can be characterized by the fact that it takes gonads in this phase at least 14 days before the stimulus of a day length of 16 hours and a temperature of 20° is able to induce maturation. From the data given in the previous paragraph it can be concluded that it generally takes young fish a longer time to complete this last stage of development than adults. The gonadal phase at which development comes to a standstill can be compared with the gonadal phase of animals caught in the sea in December and January (see table 1) for it takes the latter animals about the same time to respond to the stimulus of 16 hours day length and 20° C.

Finally the question must be answered whether the gonads remain in the phase of standstill once it has been reached, or whether they show fluctuations in the level of development. From fig. 3 it can be seen that the lines connecting the average durations of the response-times in the different groups show pronounced fluctuations. In the adult groups there is a significant difference between the duration of the response-time of group A compared with that of B and D, and between that of group C compared with that of B and D. This means that when the animals are kept for long periods of time under 8 hours day length and 20° the gonads do not remain in one particular phase of development, but show alternative periods of development (until the phase of standstill; A and C) and regression (until the phase in which it takes them a longer time to mature; B and D).

It also appears from the figure that there is about a 90-day period between the occurrence of a phase characterized by a short response-time (A and C) and that of a phase marked by a long response-time

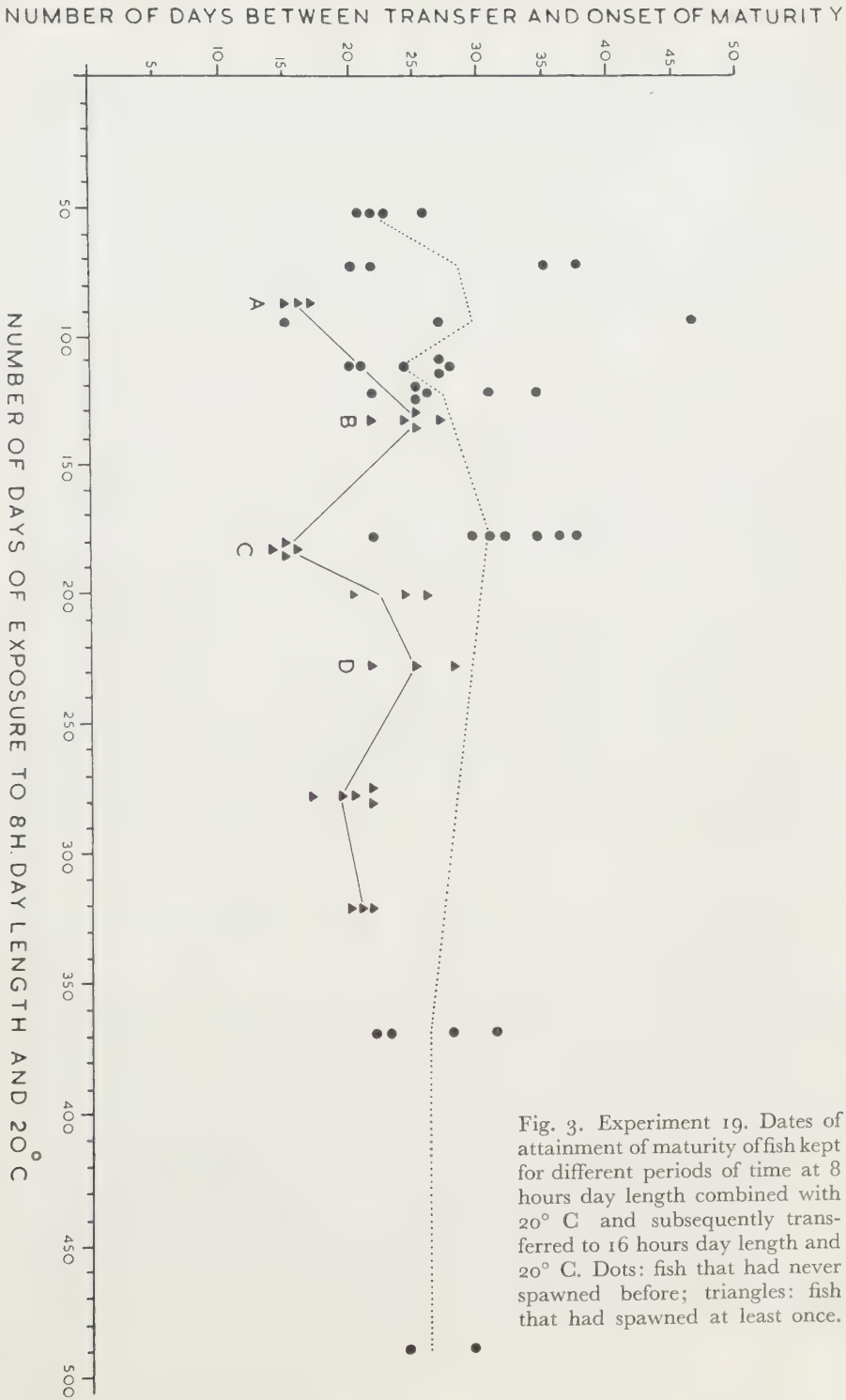

Fig. 3. Experiment 19. Dates of attainment of maturity of fish kept for different periods of time at 8 hours day length combined with 20° C and subsequently transferred to 16 hours day length and 20° C. Dots: fish that had never spawned before; triangles: fish that had spawned at least once.

(B and D). This seems to indicate that there is an inherent 90-day rhythm in gonadal development under the constant external conditions of 8 hours day length and 20° C. It is important to emphasize the fact that this rhythm occurs in gonads that do not mature.

In the young animals there are also fluctuations in the level of gonadal development but these seem to be much more irregular. It is possible that a rhythmical fluctuation does not show itself so clearly in gonads that have never reached the state of maturity.

On p. 155 the gonadal phases which might be involved will be discussed.

3. FURTHER EXPERIMENTS TO DETERMINE CRITICAL FACTORS FOR MATURATION

The experiments described in the previous sections have shown that maturation can be prevented in animals that are exposed to 8 hours day length and 20° C at a very early phase of gonadal development and that in these animals maturation can be induced by a day length of 16 hours and a temperature of 20° C. This means that a long day length (most probably above a certain threshold value), with or without a high temperature (perhaps also above a threshold value) is a crucial factor in the process of maturation.

The following experiments were carried out in order to find more factors that might be of critical importance for the attainment of maturation. It was supposed that one of these factors might be low temperature, since in nature the animals are exposed to low temperatures in winter.

a. *Exposure to a period of short day length and low temperature following the subjection to 8 hours day length and 20° C*

Experiment 20. Under -yearling animals were used that had hatched in April and May, 1954 and had afterwards been reared in the aquarium-house under almost natural conditions (see fig. 1). On July 19 (phase 0 or 1a) they were exposed to a day length of 8 hours and a temperature of 20° C. On August 28 some of these fish were subjected to a period of low temperature, while the others remained at 20°; in both groups the day length remained 8 hours. At this time the animals were in phase 1b. The period of low temperature was achieved in the following way. A decrease in temperature from 20° to 4° within 24 days, followed by an increase in temperature from 4° to 20° within 20 days. On October 6 the temperature of 20° was reached and remained constant afterwards.

The result was that neither group matured, though they were kept under this condition for more than 10 months. In Part II, experiment 22, it will be shown that there are indications that the gonads of animals exposed to the low temperature developed a little further then those of the fish which had remained at a constant temperature of 20°, though they did not mature.

The conclusion from this experiment is that in animals previously exposed to 8 hours day length and 20° C a period of low temperature combined with a short day length is not able to induce maturation.

b. *Exposure to a period of short day length and low temperature prior to subjection to 8 hours day length and 20° C*

Experiment 21. The animals were under-yearling fish that had hatched in April and May, 1955 and had subsequently been reared in the aquarium-house under almost natural conditions (see fig. 1). On August 5 some of the animals were subjected to a constant temperature of 20° C, while the others were subjected to a period of low temperature; in both groups the day length was 8 hours. In this case the majority of animals were in phase 1a. The period of low temperature consisted of a decrease in temperature from 15° to 4° within 46 days, followed by a period of 26 days during which the temperature remained 4°. At the end of the latter period the temperature was gradually raised to 20° within 41 days. The latter temperature was reached on November 26 and remained constant afterwards.

The result of this experiment was that none of the 7 animals in the group that had received 8 hours day length and a constant temperature of 20° matured during the following 6 months. When they were transferred to 16 hours day length at the end of this period (the temperature remained 20°) they matured on the average 31 days after the transfer (range 29–36).

However, in the group that had been exposed to 8 hours day length and a period of low temperature 7 out of the 9 fishes matured on the average 13 days (range 9-18) after November 26, i.e. the day on which the temperature had become again 20° C. The two remaining animals did not mature during the following 2 months when the experiment was terminated.

The conclusion from this experiment is that when young fish in a very early phase of gonadal development are first exposed to a period of short day length (8 hours) and low temperature (4°), a subsequent subjection to 8 hours day length and 20° is no longer able to prevent maturation in the majority of fishes. It cannot yet be decided here whether the response was due to the action of the low temperature alone, or whether the joint action of the short day length and low

temperature was responsible. This subject will be dealt with in the following chapter, where it will be shown that it is the latter joint action which exerts the favourable influence.

The data show that also a period of short day length and low temperature can be a crucial factor in the process of maturation.

The above experiments were made with under-yearling fish and since their response might be different from that of adults, it was decided to repeat the experiment with fully grown fish. Unfortunately the experiment had to be discarded two months after its beginning owing to a failure of the electric clock regulating the day length. No opportunity was then available to start a new experiment. But there is other evidence that also in adult Sticklebacks a period of short day length and low temperature exerts a favourable influence on the process of maturation. The experiment giving the evidence will be described in the following chapter (no. 23, group B), but its results will also be given here. Adult fish that are in a very early phase of gonadal development do not respond when subjected to 8 hours day length and 20°. But when they have first been exposed to a period of 8 hours day length combined with a low temperature (6° for 40 days; decrease from 15° to 6° and increase from 6° to 20° achieved within 2 days) a subsequent exposure to 8 hours day length and 20° results in the maturation of 3 out of the 4 animals used. Thus exposure to a period of short day length and low temperature also exerts a favourable influence on the process of maturation in adult fish, in that 8 hours day length and 20° is no longer able to inhibit maturation. Therefore, a period of low temperature and short day length is a crucial factor in the process of maturation in adult as well as in young animals.

However, with regard to the critical importance of the period of short day length combined with a low temperature, one restriction must be made. The animals should be exposed to this external condition before a certain phase in their gonadal development. This fact is illustrated in the following experiment.

Experiment 22. Under-yearling animals were used that had hatched in April and May, 1954 and had been reared in the aquarium-house under almost natural conditions (see fig. 1). On October 9 they were subjected to a day length of 8 hours and a temperature of 6° C (the decrease and increase in temperature were effected within 4 days each). On this date the animals were in phase 1b. The period of low temperature lasted until December 22 (70 days). On the latter date they were exposed to a temperature of 20° C and a day length of either 8 or 16 hours.

The result was that the animals in the 16-hour group matured on

the average 51 days after the transfer (range 32–66). But in the 8-hour group none of the animals attained maturity within 8 months after the transfer when the experiment was terminated.

This result is opposite from that obtained in experiment 21 in which under-yearling fish were exposed to a period of short day length and low temperature in August and subsequently matured. The main difference between the animals of the two experiments is that those of experiment 21 were exposed to this condition at an earlier phase of gonadal development (August) than those of experiment 22 (October).

Some objection can be raised against comparison of the results of both experiments. The duration of the period of low temperature, the speed at which the low temperature was reached and the low temperature itself were not the same in both experiments. However, in the next chapter it will be shown that the effect of the conditions as described in experiment 21 and 22 is the same when animals of the same initial phase of gonadal development are used. Thus both are able to exert a favourable effect or both are ineffective, depending on the gonadal phase at the beginning of the experiment. Comparison of the results of experiments 21 and 22 is therefore justified.

In the next chapter it will be shown that also in adult fish it depends on the gonadal phase at the beginning of the experiment whether a period of short day length and low temperature will be effective or not. The favourable influence of this external condition is restricted to a certain, rather early, phase of gonadal development. Later phases are no longer sensitive to this external condition. In experiment 20 a similar effect was demonstrated.

To simplify discussion of the problem the early phase, in which a period of short day length and low temperature is able to exert a favourable influence, has been named phase 1a, while the later phase that is no longer sensitive to the above condition, will be called 1b. Gonads in either phase 1a or 1b cannot be induced to mature under 8 hours day length and 20° C (see experiments 16, 17, 18, 19, 20, 21 and 22).

In this chapter two crucial external conditions for maturation have been demonstrated, namely 16 hours day length combined with a temperature of 20° C and 8 hours day length combined with either 4° or 6° C. As was mentioned already on p. 119 similar results might have been obtained with other day lengths and temperatures, but only when they are above (or below) certain threshold values.

In neither of the cases, however, is it known what the exact threshold values are. In the following discussions the two critical conditions will be described in more general terms by speaking of a period of long day length combined with a high temperature, and of a period of short day length combined with a low temperature.

4. DISCUSSION

a. *Critical external factors timing the onset of the breeding season*

In the previous sections it has been shown that in the 3-spined Stickleback there are two sets of external conditions that are critical factors in the process of maturation. This does not mean that the latter process depends on the action of both conditions; the presence of one of them is sufficient. In nature, however, it seems that both conditions may play an important rôle in the timing of the onset of the breeding season.

In nature the Sticklebacks are exposed to a period of short day length and low temperature in autumn and winter, while in spring they experience a period of long day length and high temperature. It seems, therefore, that the timing of the onset of the breeding period is secured in a double way. It is supposed that usually the animals will be exposed to the period of short day length and low temperature (autumn) in the right phase of gonadal development, namely phase 1a. Under influence of the above conditions the animals come into phase 2 of gonadal development and once this phase is reached maturation will inevitably occur, even if the day length remains short and the temperature rather low (see table 5).

If for some reason the animals are exposed to the period of short day length and low temperature in phase 1b, this would mean that the stimulus of the external condition would be ineffective. In this case, however, the fact that in the early months of the year the day length becomes long and the temperature high means that maturation would still be induced (see table 5).

Besides the above mentioned conditions for the process of maturation it is possible that there are still more critical factors, though in the case of the Stickleback only a few observations are available. Some factors which have been claimed to play a critical rôle in the timing of the breeding season in other Vertebrates will be discussed here with respect to their influence in the Stickleback.

Food

Between 1900 and 1930 it was thought that the quantity of food determined the onset of the breeding season. But, though it is true that without a sufficient amount of suitable food breeding cannot be successful, there is still no conclusive evidence that seasonal variations in the quantity and quality of food are critical factors in the process of maturation (for a definition of maturation as it is used here see p. 140).

With regard to the amount of food available it can be said that this factor most probably does not play a rôle in the timing of the breeding season in the Stickleback. Animals caught in their natural

habitat at different times in the year always looked very well fed. In experiment 19 in which maturation was inhibited by 8 hours day length and 20° C for over one year the animals were always given a sufficient amount of live food but, notwithstanding the presence of abundant food, maturation did not occur in these animals. CRAIG-BENNETT (1931), also working with the 3-spined Stickleback, came to the same conclusion.

However, in female birds there is some evidence that the very last phases of follicle development (phase of rapid growth of the eggs) might depend on the presence of abundant food (CLAVERT, 1953). In the Stickleback it is known that regular oviposition only occurs when the females are fed abundantly, though it is not known whether relatively little amounts of food really inhibit oviposition.

It can be said that there is evidence that in some species the quantity of food determines whether the very last phase of maturation of the eggs will take place or not. In males the formation of ripe sperm is much less dependent on the quantity of food.

It is possible that the quality of food might play a critical rôle. BISSONNETTE (1933) demonstrated that in the Starling a diet poor in proteins, fats, vitamins and mineral salts caused only a slight response of the gonads to red light compared with the effect in animals with a diet containing the above substances. In the Sticklebacks there is no indication that the quality of food is important. Animals kept for over one year under 8 hours day length and 20° C without attaining maturity were fed a variety of food. In summer they were fed live *Daphnia*, in autumn live *Tubifex*, in winter *Tubifex* or ground cooked liver and in spring again *Tubifex* or *Daphnia*. Notwithstanding this change in the quality of food the fish did not mature. However, more data are needed before a conclusive statement can be made on the rôle of the quality of food in the process of maturation.

Light

The rôle of the daily photo-period has already been discussed. Other light factors that might play a rôle in maturation are seasonal variations in the intensity and the quality of light. In Chapter IV it has been shown that intensities above 25–30 f.c. do not accelerate gonadal development any more than intensities of 25–30 f.c., though lower intensities did have a retarding effect. However, in nature such low intensities do not occur except at very restricted times of the day. Usually the light intensities are much higher, even on the dullest days in mid-winter. In the Stickleback, therefore, the change in light intensity in the pre-breeding season cannot be a critical factor for maturation. In birds it has also been demonstrated that only un-

naturally low intensities of light have a retarding effect on gonadal development (see review Benoit and Assenmacher, 1953b).

With regard to the effect of the quality of light, i.e. the spectral composition, no data are available in fishes except for the rather crude experiments with Sticklebacks described in Chapter IV. The different qualities of light that were used there had no influence on gonadal development. In birds it has been shown that white and red light are most effective, while green light has no effect or a somewhat retarding influence (see review Benoit and Assenmacher, 1953b). These data, however, give no indication that in nature the relatively slight change in the composition of the light might be a crucial factor for the process of maturation.

In Chapter V it was mentioned that the gradual increase in day length as such might be of critical importance for the timing of the onset of spermiogenesis in birds. In the Stickleback this might also be true, though no experiments have been made yet on this subject. It was demonstrated, however, that the total amount of light received is of much greater importance for gonadal development than the gradual increase as such.

Migration to the sea

Some fishes, like the Stickleback, carry out migrations to and from the sea that are correlated with either gonadal development or regression (see Part II). It is possible that salt water is of critical importance for maturation. In the 3-spined Stickleback this is undoubtedly not true. Animals kept the year round in fresh water under natural conditions attain maturity at the same time as animals that have carried out the migration and have spent the winter in the sea. To give a few figures: animals of the former group when transferred to the aquarium-house in April (conditions almost similar to those in nature) matured on the average on April 14 (range 4–19). Animals caught on the sea-side of a sluice in April and transferred to the aquarium-house on the same date as the former group, matured on the average on April 19 (range 15–22). The difference between the two groups is not significant.

However, migration to the sea might be favourable for the general condition of the fish, for it has been observed that animals living in sea-water after the breeding season are much less susceptible to all kinds of diseases than those kept in fresh water.

Psychological factors

So called "psychological factors" also play a rôle in reproduction. Examples of such factors are immediate environment and the nesting site which must both be suitable. Further, the stimulus of the presence

of animals of the same or opposite sex might be important in some species (see BEACH, 1948; BULLOUGH, 1951). These factors only seem to play a rôle in the very last phases of maturation, particularly in the female, and might therefore be called critical factors in the process of maturation. In the Stickleback, however, the absence of nesting-sites and the environment do not effect maturation in either the male or the female. No experiments have yet been made on the influence of stimuli coming from animals of the same or opposite sex on maturation in this species.

Other factors that have been reported to play a (critical) rôle in maturation of some species of Vertebrates are moonlight, tidal-currents, rainfall and its influence on humidity, presence of green vegetation etc. These conditions will not be discussed here because it is very unlikely that they will be of critical importance for maturation in the 3-spined Stickleback.

To end this discussion on critical factors it is emphasized again that if more than one critical factor is found, this does not mean that maturation depends on the action of all these factors, but that it is possible that each one of them would be sufficient to secure maturation. However, the presence of more than one critical factor seems to have the function of safeguarding breeding in nature under all circumstances.

b. *Does an intrinsic factor or rhythm cause the onset of the breeding season?*

BAKER and BAKER (1936) used the term intrinsic rhythm "to indicate a tendency on the part of any animal to behave rhythmically in relation to reproduction independently of the environment". Undoubtedly the word "independently" in this definition means "independently of seasonal fluctuations in external factors, even though these factors themselves may be causally involved". This is the sense in which the term intrinsic rhythm or factor is used in this paper.

Now the foregoing experiments with Sticklebacks have shown that when fish in phase 1a or 1b are exposed to the constant external condition of 8 hours day length combined with 20° C they do not attain maturity. Maturity is induced only under either of the following two conditions: *a.* exposure in phase 1a to 8 hours day length combined with a low temperature, followed by a high temperature (20° C) at the same day length, *b.* exposure to a long day length combined with a high (perhaps also a low) temperature.

This means that in the three-spined Stickleback maturity can only be attained under the influence of certain external factors. In the absence of these external factors no intrinsic factor is able to induce maturity.

A number of authors claim to have demonstrated the presence of an intrinsic factor or rhythm timing the onset of the breeding period, but their experiments are not conclusive. BULLOUGH (1940) working with minnows and HILL and PARKES (1934) working with ferrets subjected these animals in mid-winter to complete darkness (except for a few minutes of light during feeding) and observed that the animals nevertheless came into breeding condition. However, the result of such an experiment cannot be conclusive with respect to the presence of an intrinsic factor, because some gonadal development had already taken place between the end of the previous breeding period and the beginning of the experiments in mid-winter. It is possible that the latter part of gonadal development can take place "by itself" or under the influence of very minor external stimuli. This assumption is supported by the fact that MARSHALL and BOWDEN (1934) who made a similar experiment with ferrets but started it earlier, found that in this case the animals failed to come on heat when kept in incomplete darkness.

The observation that blind animals show a seasonal cycle of reproduction is also not conclusive, since it is possible that light may reach the hypothalamus via the optic nerve or tissues in the orbita (BENOIT and ASSENMACHER, 1953 a, b; 1955). Grouse and pheasant kept continuously in the dark fail to attain maturity (CLARK *et al.*, 1937).

The transportation of animals from one hemisphere to the other has provided valuable information on the presence of an internal rhythm regulating the breeding season. MARSHALL (1936, 1937); BEDFORD and MARSHALL (1942), and BULLOUGH (1951) have summarized the data available. The general conclusion that can be drawn from these data is that many animals possess an internal rhythm, though in only a very few cases does this rhythm seem to be strong enough to cause the onset of the breeding season without any influence of external factors. In these cases the animals after transportation, did not adapt themselves to the new external conditions but continued breeding at the original time of the year. However, in most cases the internal rhythm was rather weak and could be reversed by the external conditions, either immediately after transportation or after one or more seasons.

Recently the presence of an intrinsic rhythm regulating the onset of the breeding season in a bird has been conclusively shown by BENOIT and ASSENMACHER (see discussion of paper by MARSHALL, 1955). They placed immature ducks in complete darkness for 20 months. It was observed that the ducks became mature (though considerably later than animals under normal circumstances), while spontaneous regression and regrowth of the gonads occurred.

Though the presence of an intrinsic rhythm has been shown in a

number of cases, it is inconceivable that such a rhythm would be the sole factor regulating the onset of the breeding season. BAKER and BAKER (1936) calculated that if this rhythm were incorrect to the minute extent of 6 minutes per year, then if the species were breeding in the spring at the end of the last glacial epoch in the northern hemisphere, it would be breeding at precisely the wrong season of the year (autumn) now.

Therefore, in all animals the annual breeding season must be "set in time" by external conditions.

c. *Alternative phases of development and regression in the gonads of immature Sticklebacks kept under constant external conditions*

In section 2 it has been shown that rhythmical changes occur in the gonads of fish kept long for periods of time under 8 hours day length and 20° C without attaining maturity. Also in the Hake (*Merlucius merlucius*) seasonal changes occur in the ovaries of immature fish (HICKLING, 1935). Partial activation and recession of the gonads is also known in some gallinaceous birds (HIATT and FISHER, 1947; MILLER, 1948). These observations indicate that completion of the process of maturation is not essential for the occurrence of rhythmical changes in the gonads. In the case of the Hake the changes might have been caused by external and or internal factors, but in the case of the Stickleback they must have been caused by an intrinsic factor alone or by the co-operation between intrinsic and external factors, since the external conditions were constant.

On the basis of the results obtained in experiments 16 and 17 (slight gonadal development under 8 hours day length and 20° C) and experiment 20 (development from phase 1a to 1b) it is suggested that under constant external conditions of 8 hours day length and 20° C gonadal development fluctuates between phases 1a and 1b.

VII. THE INFLUENCE OF VARIOUS DAY LENGTH AND TEMPERATURE CONDITIONS AT DIFFERENT TIMES IN THE PERIOD FOLLOWING TERMINATION OF THE BREEDING SEASON

In Chapters III and IV the influence of temperature and day length on gonadal development in the period of 3 months before the onset of breeding, i.e. on the last phases of development, has been described. In the present experiment the effect of various external conditions on gonadal development in the period following the termination of the breeding season, i.e. on the first phases of development, is studied.

For a better understanding of the experiment described below a few remarks must first be made on the experimental procedure. The experiment consisted of two parts, (*a*) the "experimental part" in which the animals were exposed to various external conditions during a certain period of time, followed by (*b*) subjection to two different "test-conditions" (namely 8 hours day length and 16 hours day length, each combined with a temperature of 20° C) in which the phase of gonadal development reached under the experimental conditions could be determined. The reason for testing the phase of gonadal development by the above two conditions was the following. It has been shown in previous chapters that the early phase of gonadal development (phase 1) does not respond to 8 hours day length and 20°, while phase 2 does. Both phases respond to the stimulation of 16 hours day length and 20°, but it takes animals in phase 1 a longer time to mature than those in phase 2. Now, in the present experiment animals in phase 1 will be exposed to various external conditions for a certain period of time, during which gonadal development may take place, but no maturation occurs. When development from phase 1 to phase 2 has taken place under these experimental conditions, for example, the animals would respond to the test-condition of 8 hours and 20°. In this case the duration of the response-time to the test-condition of 16 hours and 20° would be shorter than if the fish had remained in phase 1. In this way subjection to the test-conditions can give information on gonadal development that may have taken place under the various experimental conditions.

1. THE INFLUENCE OF VARIOUS EXTERNAL CONDITIONS ON DIFFERENT PHASES OF GONADAL DEVELOPMENT

Experiment 23. The animals used were adult males that had reached maturity between February and April, 1954, under experimental conditions, (thus one or two months prior to the natural breeding season, see also p. 162), and had afterwards been maintained in the basin outside the laboratory (for conditions see fig. 1). On August 23 the following series of experiments were started.

Group A: Day length 8 hours; temperature 20° C.
Group B: Day length 8 hours; temperature 6° C.
Group C: Day length 16 hours; temperature 6° C.
Group D: Natural conditions in basin outside the laboratory (see figure 1).

On October 6 (i.e. after 45 days) the gonadal condition of the animals in the different groups was tested by exposing them to a day

length of 8 hours and a temperature of 20° C, and to 16 hours day length and 20° C. The decrease and increase in temperature in the experiments was in all cases achieved within two days each, these being included in the period of 45 days.

To determine the effect of the different external conditions on a later phase of gonadal development a similar series of experiments was started on October 9 with animals that had been left in the basin under natural conditions until that date. The second part of these experiments (i.e. the tests) started on November 25, thus also after 45 days of exposure to the experimental conditions. The results of both sets of experiments is given in table 3. In neither of the experiments did the animals mature during the period of exposure to the experimental conditions; if maturation took place it happened only during exposure to the test-conditions.

First the results of the experiments will be discussed with respect to the effect of the various external conditions at the two different times in the season, i.e. on different phases of gonadal development.

Group A: Day length 8 hours; temperature 20° C

This experiment can be used to determine the phase of gonadal development at the beginning of the experiments, namely on August 23 and October 9, for in this case the experimental condition is similar to one of the test-conditions.

The table shows that under the test-condition of 8 hours day length and 20° no animals matured in the August group or in the October group. This means that on August 23 and on October 9 all animals were in phase 1 of gonadal development.

The table also shows that all 4 August animals responded to the test-condition of 16 hours day length and 20° (on the average 17 days after the transfer). The corresponding figure for the 4 October animals was 20 days. The difference between the duration of the response-times in both groups is significant at the 0.05 level. The result indicates that the experimental condition of 8 hours day length and 20° during the period October 9 to November 26 has exerted a somewhat unfavourable influence on gonadal development when compared with the effect of the same condition during the period August 23 to October 6. This indicates that, although the animals remained in phase 1 of gonadal development between August 23 and October 9, they nevertheless were in a different stage of development within this phase, for the reaction to the same experimental condition (8 hours day length and 20°) was different. The phase of gonadal development on August 23 has been named 1a and that on October 6, 1b.

TABLE 3

The influence of various day length and temperature conditions on early phases

Group	Experimental treatment	Period of treatment	Initial phase of gonadal development[1]	Test-condition: 8 h. day length; 20° C number of fish responding	not responding	duration (days) of response-time average	range
A	8 h. day length	23 Aug.– 6 Oct.	1a	0	4	—	—
	temp. 20° C	9 Oct.–26 Nov.	1b	0	4	—	—
B	8 h. day length	23 Aug.– 6 Oct.	1a	3	1	17	16–18
	temp. 6° C	9 Oct.–26 Nov.	1b	1	3	11	—
C	16 h. day length	23 Aug.– 6 Oct.	1a	0	5	—	—
	temp. 6° C	9 Oct.–26 Nov.	1b	6	2	15	8–31
D	Natural	23 Aug.– 6 Oct.	1a	0	10	—	—
	conditions	9 Oct.–26 Nov.	1b	4	2	45	27–79

[1] The physiological condition of the fish used here has proceeded further than would as the fish had been exposed to special artificial external conditions before the experime

Group B: Day length 8 hours; temperature 6° C

Table 3 shows that in the test with 8 hours day length and 20°, 3 out of the 4 August-animals matured (on the average 17 days after the transfer). The last animal did not mature within 4 months after the transfer, when the experiment was terminated. The test with 16 hours day length and 20° shows that all 3 animals used matured (on the average 16 days after the transfer). In the October animals only one of the four matured under the test-condition of 8 hours day length and 20°. When exposed to the test-condition of 16 hours day length and 20° all 4 animals matured (average response-time 24 days).

The conclusion that can be drawn from these results is that in August (phase 1a) a short day length and low temperature stimulates gonadal development, for the majority of fish possessed gonads in phase 2 on October 6. This stimulating effect of a period of short day length and low temperature has already been shown in Chapter VI. However, in the October animals (phase 1b) only one of the fish responded to the same external condition. This means that phase 1b cannot be favourably affected by a short day length and low temperature. There is no significant difference between the duration of the response-times to the test-condition of 16 hours day length and 20° in the August and October groups.

The result of the experiments in this group confirm that of the first group (A) in that it also shows that in August the animals are in a different phase of gonadal development than in October (phases 1a and 1b respectively).

nadal development.

Test-condition: 16 h. day length; 20° C number of fish responding	not responding	duration (days) of response-time average	range
4	0	17	15–18
4	0	20	18–21
3	0	16	15–17
4	0	24	9–33
8	0	23	14–39
8	0	13	7–28
10	0	21	12–35
8	0	29	22–32

rmal at this time of the year, rted.

Group C: Day length 16 hours; temperature 6° C

Table 3 shows that in the August group (phase 1a) the above external condition has no favourable effect, for the fish do not respond to the test-condition of 8 hours day length and 20°. When exposed to the test-condition of 16 hours and 20° all 8 animals matured (on the average 23 days after the transfer).

However, the above condition does have a favourable influence on the animals in the October group (phase 1b), for 6 out of the 8 animals matured on the average 15 days after the transfer to the test-condition of 8 hours and 20°. This means that the majority of animals in phase 1b had developed to phase 2 under the influence of the above condition. This difference in reaction of animals in phase 1a and 1b is supported by the fact that the latter (October group) responds earlier to the test-condition of 16 hours and 20° (on the average 13 days after the transfer) than the former (August group, 23 days). The difference between these response-times is significant ($P = 0.038$).

Group D: Natural conditions

In this case the results obtained in August and in October cannot be compared so well, because the external conditions are different at these times of the year. Between August 23 and October 6 the day length decreases from about 14–11 hours and between October 9 and November 26 from 11–8 hours. In the first period the temperature decreases from about 14–11° and in the second period from about 11–6° (see fig. 1). Nevertheless conclusions can be made on the phase of gonadal development of animals under natural conditions on October 6 and November 26.

On October 6 the animals living under natural conditions in the basin outside the laboratory were still in phase 1, for they did not respond to the test-condition of 8 hours and 20°. When exposed to the test-condition of 16 hours and 20° they responded on the average 21 days after the transfer (see table 3).

On November 26 the animals living in the basin under natural conditions had reached phase 2 for the majority responded to the test-condition of 8 hours and 20° (on the average 45 days after the transfer). These animals matured on the average 29 days after the transfer to the test-condition of 16 hours and 20°. The difference in

response-times of the October and November groups to the test-condition of 16 hours and 20° is significant (P = 0.006). This also indicates that under natural conditions the gonads show a development from phase 1 to phase 2 between October 6 and November 26.

Summarizing the results described above it can be said that the animals in the August group, as well as those in the October group were in phase 1 at the beginning of the experiments (A). Detailed investigation showed that the August animals were in phase 1a and the October fish in phase 1b of gonadal development (A, B and C). In phase 1a gonadal development can be stimulated by 8 hours day length and 6° to reach phase 2. However, phase 1b does not react to the same stimulus by developing to phase 2, but remains stationary (B). Animals in phase 1a do not respond to the stimulus of 16 hours day length and 6° by developing to phase 2, but this does take place when fish in phase 1b are exposed this condition (group C). Natural conditions in August and September (see above) are not able to stimulate development from phase 1a to phase 2, but natural conditions in October and November (see above) induce development from phase 1b to phase 2 (D).

The results can also give information concerning which one of the four external conditions used in these experiments is most favourable for the development of phases 1a and 1b. Data on this subject can be obtained by comparing the responses of each of the two phases to the four external conditions that have been used.

The most favourable condition for a quick development of phase 1a to phase 2 is 8 hours day length and 6° (see group B), for maturation under the influence of the test-condition of 8 hours day length and 20° occurs only in this group. An attempt was made to find which of the three remaining external conditions would be the next most favourable (A, C or D). But comparison of the duration of the response-times to the test-condition of 16 hours day length and 20° did not reveal any significant differences.

For a quick development from phase 1b to phase 2 two conditions are favourable, namely a day length of 16 hours combined with a low temperature and the natural condition during October and November (groups C and D respectively). In C 75 % of the animals responded to the test-condition of 8 hours day length and 20°, while this figure was 66.4 % in group D. The difference in the duration of the response-times to this test-condition is significant (P smaller than 0.004) and this is also true for the difference in the response-times to the test-condition of 16 hours day length and 20° (P smaller than 0.003). This means that the most favourable condition for a quick development from phase 1b to phase 2 is a day length of 16 hours combined with a low temperature.

The next most favourable is the natural day length during October and November (day length decreasing from 11–8 hours; temperature decreasing from 11–6°). The next best might be 8 hours day length and 6°, for one out of the four animals responded to the test-condition of 8 hours day length and 20°, while none of the fish exposed to 8 hours and 20° (experimental condition in group A) responded to this test-condition. The difference in response-times of both groups (8 hours 20° and 8 hours

6°) to the test-condition of 16 hours day length and 20° is not significant. Therefore, it seems that there is hardly any difference between the effects of both conditions; they are equally unfavourable. It must be stated here that 16 hours day length and 20° is able to induce development in all gonadal phases described here. However, it cannot be concluded whether this condition would be more favourable for development of any of the phases than the conditions A, B, C and D.

It must be emphasized that the finding of the most favourable external condition for quick development of phases 1a and 1b as described here only holds for the four different conditions used in this experiment. It might very well be possible that there are still more external contitions which are favourable (or unfavourable) for gonadal development of certain phases.

The main conclusion that can be drawn from the results of this set of experiments is that whether an animal will respond to a given external condition or not depends on the phase of gonadal development. Different gonadal phases often need different external conditions to develop.

2. THE COMBINED ACTION OF A CERTAIN DAY LENGTH AND A CERTAIN TEMPERATURE AS A STIMULATING OR INHIBITING CONDITION FOR GONADAL DEVELOPMENT

In Chapter VI and also in experiment 23 it was shown that phase 1a can be stimulated to develop to phase 2 by 8 hours day length combined with a low temperature. Now the question arises as to whether this favourable influence must be attributed to the short day length or to the low temperature, or to the joint action of both components. This question can be answered when the results of groups A, B, and C of experiment 23 are compared.

It has been shown that, *a*. 8 hours day length together with a low temperature was effective, *b*. 8 hours day length and 20° C had no effect, and *c*. 16 hours day length and low temperature was also ineffective (effect means development from phase 1a to phase 2). These facts lead to the conclusion that it is the specific combination of the short day length and the low temperature which has a stimulating influence on gonadal development in phase 1a.

On the other hand it can also be shown that it must be the specific combined action of 8 hours day length and 20° C which inhibits development of phase 1a, for *a*. 8 hours day length and 20° inhibits development, *b*. 8 hours day length and low temperature stimulates development, while *c*. 16 hours day length and 20° also stimulates development from phase 1a to phase 2.

Neither phase 1b nor phase 2 need specific combinations of day length and temperature to develop. In phase 1b development to phase 2 can be induced by a long day length together with a low as

well as a high temperature, while this development can be inhibited by a short day length combined with a low as well as a high temperature. In this case, therefore, development from phase 1b to phase 2 depends solely on the day length. In phase 2 any combination of long or short day lengths together with high or low temperatures is able to induce development to maturity (see table 5).

It was mentioned on p. 157 that the decrease in temperature to reach the required temperature of 6° C and the subsequent increase to reach the required temperature of 20° C was achieved in the present experiment within two days each. Now it cannot be decided whether it has been the period of low temperature as such, or the decrease or increase as such that has exerted the favourable influence on phase 1a in combination with the day length of 8 hours. It can only be concluded that the rate of decrease or increase to which the animals have been exposed does not affect the results. For in experiment 21 the decrease and increase in temperature was achieved very slowly while in experiment 23 it was accomplished within two days; the effect of both methods was the same.

3. DISCUSSION

The first point that must be discussed here is the question whether the effect on gonadal development of the different external conditions to which the animals have been exposed in the foregoing experimental series will also play a rôle in nature.

Before this question can be answered it is necessary to make a statement on the phase of gonadal development of the animals used in experiment 23 in August and October. It was mentioned that the animals came from a group that had been artificially induced to mature between February and April, i.e. one or two months prior to the breeding season in nature. It can be assumed, therefore, that the whole cycle of the fish, including the development during the period following breeding, took also place in the experimental animals one or two months earlier than in nature. However, it must be remembered that the external conditions to which the fish were exposed during the period following breeding were not similar to those to which the animals in nature are exposed during this period. This might have caused differences in the rate of development in the period following breeding in the experimental animals, which in turn may mean that the difference in the gonadal cycle of the experimental fish and those in nature has been either more of less than one or two months.

However, there is another way to determine which animals in nature must be compared to the experimental fishes in August and October.

It has been shown that the August and October animals were all in phase 1 of gonadal development (the distinction of phases 1a and 1b will be left out for a moment). In table 1 and 2 it can be seen that in December, January and February some of the animals do not respond to the stimulus of 8 hours day length and 20° C, while others do. Therefore, the former animals at that time were still in phase 1, while the latter were already in phase 2. Since the number of animals that does not respond decreases from December until March it can be assumed that the change from phase 1 to phase 2 takes place in these months. This means that the gonadal phase of the animals used in the present experiment can be compared with that of animals in nature between December and February. This fact will be the basis for the following discussion on the effects of various external conditions on gonadal development of animals living in their natural habitat during the period following breeding.

It has been shown that in phase 1a exposure to a short day length and a low temperature results in quick development from phase 1a to phase 2. It can be assumed that phase 1a will be reached in nature around November. At this time of the year the day lengths are short while the temperature is also low (see fig. 1). It is, therefore, very likely that in nature this condition will also exert a favourable influence on the development of gonadal phase 1a, with the result that the fish reach phase 2 in the months following November. Tables 1 and 2 show that in nature phase 2 is indeed reached by some of the animals.

With regard to phase 1b (the later stage of phase 1) it has been shown that this stage can no longer be favourably affected by a short day length and a low temperature. Now what consequences can this phenomenon have on gonadal development in nature? It is possible that in the period following breeding development takes place very rapidly in some of the animals. This would mean that before the day lengths become short and the temperatures low the gonads of these fish have already reached stage 1b. In this case the subsequent period of short day length and low temperature will have no effect, i.e. the gonads do not develop to phase 2. This development can only take place when the day lengths become relatively long and the temperatures relatively high, thus in nature sometime in January or February. In this way the phenomenon that phase 1b does not react to the stimulus of a short day length combined with a low temperature may have the function of preventing maturation at an untimely season of the year in fishes that, for some reason, had developed very quickly in the period following breeding. This also might be the reason that between December and February there are still animals that have not reached phase 2 (tables 1 and 2).

In case the development of the gonads would, for some reason, proceed very slowly in the period after breeding it might happen that phase 1a would be reached later than December and would no longer be able to profit by short day lengths and low temperatures. However, in this case the relatively long days and high temperatures in the early months of the year would ultimately cause maturation, because it has been shown that this condition induces development in all phases, from phase 1 to maturity.

This reasoning holds for gonadal development of adult fish, since the experiments were made with adults. But in Chapter VI it has been shown that young animals also respond to the influence of a short day length and low temperature by showing gonadal development from phase 1a to phase 2 (experiment 21). Again young animals do not respond anymore to the stimulus of a short day length and low temperature later in their development (phase 1b; see experiment 20). Therefore, it is justified to assume that in nature the timing of the gonadal phases 1 and 2 in under-yearling fish will be regulated in the same way by the external conditions as in adult animals. The discussion given here on the regulation of the breeding season in nature is a more extended version of the discussion given on p. 150.

It will be noticed that in experiment 21 the young fish had already reached phase 1a in August, while in nature this stage should be reached only around November, in order to profit by the short day length and low temperature at that time of the year. But it has been noticed that under-yearling fish reared under laboratory conditions grow much faster than in nature (most probably as a consequence of abundant food). This is shown, for example, by the fact that in nature under-yearling fish caught in November measured on the average 3.5 cm in November, 3.8 cm in December, and 3.9 cm in January, whereas the fish used in experiment 21 already measured on the average 3.6 cm in August, while those used in experiment 20 measured 3.4 cm at that time. Therefore, there is no reason to assume that the above reasoning would not hold for under-yearling fish.

The second point that must be discussed here is the fact that in the literature so many statements can be found to the effect that certain external conditions do not play a rôle in the timing of the onset of the breeding season, while this conclusion is based on experiments made in a very restricted time of the year, i.e. made with animals being in perhaps only one phase of gonadal development. The experiments described in this and previous chapters have conclusively shown that the gonadal response of an animal to a given external condition depends on the phase of gonadal development at the time of the experiment. It has also been shown that different phases of gonadal development often need different external conditions to develop, while it has been proved that development sometimes depends on a particular combination of day length and temperature. These facts show that

one must be very careful in stating that a certain external condition has no influence on the timing of the breeding season.

It seems to have escaped the attention of many workers in this field that the timing of the breeding season begins as soon as the gonads start to develop (in the under-yearling fish) or to regenerate (in the case of adults after the breeding season). CRAIG-BENNETT (1931) working with the 3-spined Stickleback and TURNER (1919) with the Perch (*Perca perca*) have shown that in these animals spermiogenesis begins almost immediately after the end of the breeding season. In birds gonadal recrudescence also takes places directly after the end of the breeding season (revival of singing in August and September). This means that also in birds the timing of the breeding season starts immediately after the end of the previous breeding period. This again shows that conclusions on the timing of the breeding season based on experiments made in mid-winter and early spring may reveal only part of the picture.

The fact that the timing of spring-breeders has only been studied with regard to long day lengths and high temperatures, has led to the wrong conclusion in the literature that in these animals the timing-mechanism has become adapted to typical spring conditions in order to produce the offspring at the most favourable time of the year. In the case of autumn breeders it is said that the timing-mechanism of these animals has become adapted to typical autumn conditions in order to produce offspring at the most favourable time of the year for these species. In the Stickleback, a typical spring-breeder, it has been shown in Chapters III and IV that the gonads in the early months of the year indeed respond best to spring conditions, namely a long day length and a high temperature. But in the present chapter it has been shown that other phases of gonadal development respond best to a short day length and a low temperature (autumn- and winter-condition) and that this phenomenon too has a function in the timing of the breeding season. Therefore, the timing of the breeding season in the Stickleback has become attuned not only to typical spring-conditions, but also to typical autumn- or winter-conditions.

The timing of the reproductive season in animals is even more complicated than is generally assumed. It involves a most delicate interaction between internal and external factors in each phase of gonadal development, from the very beginning (after the end of the previous breeding season) until the fertilization of the eggs.

VIII. INDUCTION AND PREVENTION OF MATURATION IN UNDER-YEARLING STICKLEBACKS BY MEANS OF 16 HOURS DAY LENGTH COMBINED WITH A TEMPERATURE OF 20° C

In the previous chapters a number of experiments have been described in which under-yearling fish were induced to mature by means of different external conditions (experiments 3, 7, 10, 11, 16, 17, 21 and 22). In nature Sticklebacks become mature at the end of their first year, but some of the above experiments have shown that it was possible to induce maturation in animals not older than 8 months. Now the question arose whether it would be possible to induce maturation in animals that are younger than 8 months.

1. THE REACTION OF YOUNG FISH TO 16 HOURS DAY LENGTH AND 20° C

Experiment 24. Under-yearling animals were used that had hatched in the laboratory in April and May and were afterwards reared in the aquarium-house under almost natural external conditions (see fig. 1). The experiment could not be completed within one year, owing to shortage of animals and was therefore extended over the years 1953, 1954, and 1955. The fishes were exposed to 16 hours day length and 20° C between June and March of the next year. This condition was chosen, because it is known that animals in many phases of gonadal development respond very well to this stimulus (see table 5).

Experiments were started on the following dates: June 21, September 29, and October 23, 1953; June 14, July 19, October 9, and December 22, 1954; March 1 and July 11, 1955. The results are given in fig. 4.

It follows from this figure that the youngest animal in which maturation was induced aged 3.5–4.5 months (male August 1954; length 4.6 cm). Other animals that matured at an early age were two females 5–6 months old (September 1953; 3.9 and 5.2 cm); three more females of 6–7 months old (October 1953 and 1954; November 1, 1954; lengths 4.5; 4.9 and 5.0 cm respectively) and one male and one female of 7.5–8.5 months (November 1953 and 1955; length 5.0 and 5.1 cm respectively). It seems that 3.5–4.5 months is the minimum time young animals need to mature under 16 hours day length and 20° C.

The figure also shows that some of the animals submitted to 16 hours day length and 20° at a very early date, namely in June and July, did not reach maturity, even though maintained under this condition for over one year. Only 43 % of the group of June 21, 1953 matured, while the corresponding figure for the group of June 14, 1954

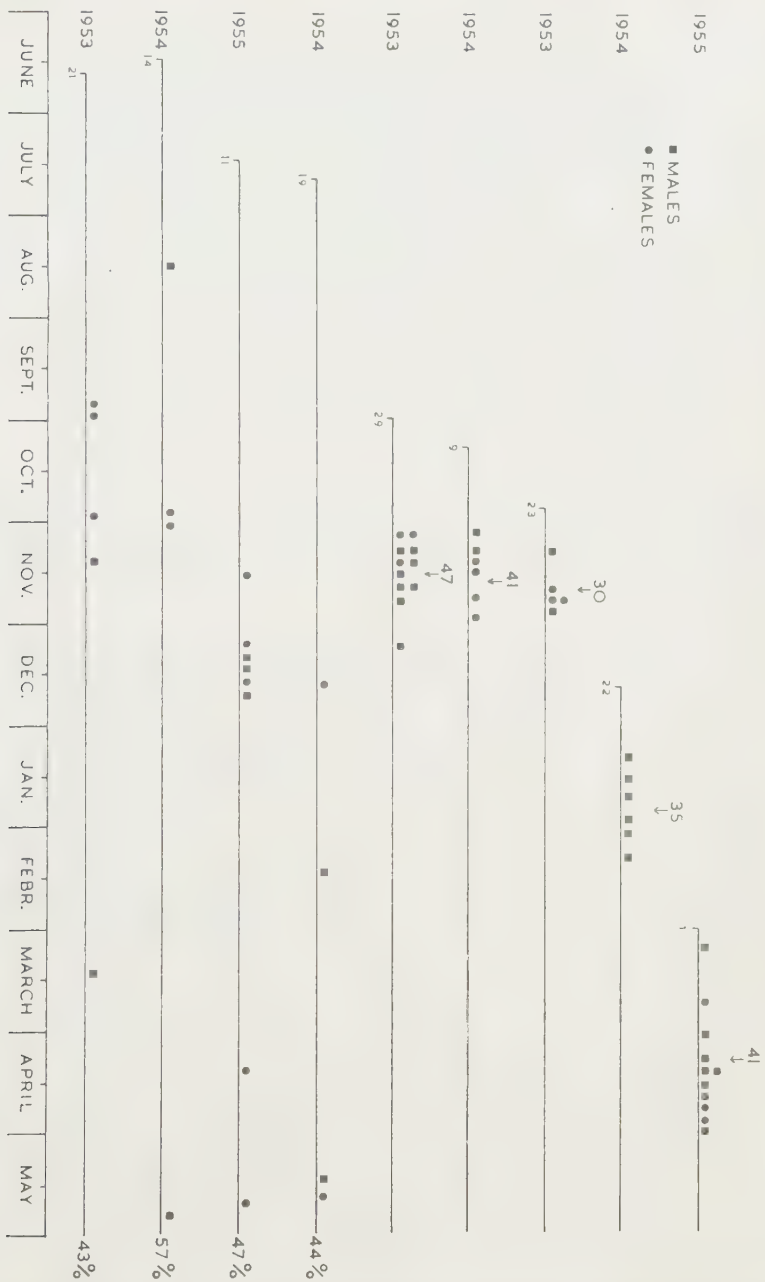

Fig. 4. Experiment 24. Dates of attainment of maturity by young fish hatched in April and May and exposed to 16 hours day length and 20° C at various times of the year. For explanation of arrows and figures see fig. 2.

TABLE 4

Growth rates of fish hatched in April and May and exposed to 16 hours and 20° C at various times of the year.

Year	*Date of beginning of experiment*	*Number of fish*	*Initial length in cm*		*Initial length of largest fish*	*Percentage of ripe fish within 1 year*	*Length at maturit*	
			average	*range*			*average*	*rang*
1954	June 14	7	3.3	3.0–3.7	3.2–3.7	57	4.9	4.6–5
1953	June 21	12	3.0	2.2–3.6	3.2–3.6	43	4.8	3.9–5
1955	July 11	17	3.0	2.6–3.4	3.3–3.4	47	5.4	5.1–5
1954	July 19	9	2.6	2.4–2.8	2.6–2.8	44	5.7	5.1–6
1954	Sept. 29	11	4.1	3.9–4.5	—	100	4.7	4.3–5
1954	Oct. 9	6	3.9	3.5–4.7	—	100	4.6	4.2–5
1953	Oct. 23	5	4.3	3.9–4.8	—	100	5.1	4.9–5
1954	Dec. 22	6	4.6	4.4–4.9	—	100	4.8	4.6–5
1955	March 1	11	5.1	4.7–5.6	—	100	5.3	4.8–6

was 57 %; for the group of July 11, 1955, 47 %; for the July 19 group, 1954, 44 %. However, all animals subjected to 16 hours day length and 20° later than September 28 attained maturity within 2 months of the beginning of the experiment.

These results indicate that when under-yearling Sticklebacks are exposed to 16 hours day length and 20° at a very early age, this treatment inhibits maturation in at least some of the animals. Except for the fish mentioned above which matured within 8.5 months after hatching, all other animals in the groups of June and July attained maturity later than the animals that were exposed to the same condition between September and October (see fig. 4). Thus, compared with the later group, maturation was greatly retarded in the former animals.

The conclusion from these experiments is that there seems to be a critical age before which young animals cannot be induced to mature when subjected to 16 hours day length and 20° C. Below this critical age the above condition has an inhibiting influence on gonadal development. If the critical factor is indeed related to the age of the fish at the beginning of the experiment then it seems that 3–4 months is the crucial age. However, depending on whether the external conditions have been favourable or not for growth, the length of the fish of 3–4 months old may vary considerably. It seems possible that whether or not a young fish will respond to 16 hours day length and 20° depends on its stage of development. Therefore, the length of the fish might be a better criterion for determination of the critical stage before which the animals do not respond to 16 hours day length and 20° C.

In table 4 the lengths of the animals at the beginning of the experiment are given, together with the lengths at the attainment of maturity or at the time the experiment was terminated. Unfortunately, the

ength	ength immature fish after one year	
	verage	range
	5.8	5.4–6.0
	5.8	5.5–6.0
	5.9	5.4–6.2
	5.7	5.5–6.1
	—	—
	—	—
	—	—
	—	—
	—	—

initial lengths of the animals which matured in the June and July groups is not known. Therefore, it cannot be decided whether or not it was the animals that were largest at the beginning of the experiment which attained maturity in these groups, though it does not seem unlikely. For this reason also the lengths of the largest fish at the beginning of the experiments have been given for the above groups.

The table shows that animals of less than 2.2 cm that are exposed to 16 hours day length and 20° will not mature, while it is possible that animals up to 2.6 cm will also not mature. It can be concluded that there is a critical stage in the development of the young Sticklebacks during which no maturation can be induced by 16 hours daylength and 20° C. At this stage of development the gonads are in phase 0.

The following experiment will show even more convincingly the inhibiting effect of 16 hours day length and 20° C on gonadal development in young animals that are in phase 0.

Experiment 25. Under-yearling fishes were used that had hatched in April and May, 1955 and had afterwards been reared in the aquarium-house under almost natural conditions (see fig. 1). On July 11 the animals were exposed to a temperature of 20° C, while the day length was either 8 or 16 hours. The initial length of the fish in both groups was 3.0 cm (range 2.6–3.4).

The result obtained in the 16-hour group has already been given in fig. 4. At the end of December 6 out of the 17 animals (35 %) had attained maturity, while two more animals matured in April and May respectively. The remaining animals did not mature in this group when kept until July.

Of the 8-hour group no animals matured until November 19 (at this time only one animal in the 16-hour group had matured). On this date the animals were transferred to a day length of 16 hours, while the temperature remained 20° C. All 6 transferred animals matured; the mean duration of the response-time was 27 days (range 23–34). When the last animal in this group matured, a total of only 6 animals in the 16-hour group had matured, while 11 had not yet attained maturity.

The result of this experiment shows again that 16 hours day length and 20° C inhibits maturation in some of the animals that are exposed to this condition at a very early age. It is supposed that the animals which did not mature had not yet surpassed the critical phase (phase 0) when the experiment started. The fact that all animals that had first been exposed to a day length of 8 hours and a temperature of 20° C

and subsequently to 16 hours day length and 20° C attained maturity, means that development of phase o takes place under the former condition, while it is inhibited under the latter.

In order to allow a better understanding of the following experiments it is necessary to describe here part of the general concept on the pituitary-gonad mechanism that will be fully discussed later in Chapter XI.

There may be two reasons for the fact that young animals in phase o do not respond to a day length of 16 hours combined with a temperature of 20° C. It is known that gonadal development depends on the presence in the blood of gonadotrophic hormones secreted by the pituitary. Now it is possible that in the above case this function of the pituitary is inhibited by the particular external condition with the result that the gonads cannot develop and mature. But it is equally possible that the pituitary secretes a sufficient amount of gonadotrophins, but that under the above external conditions the gonads are not sensitive to these hormones and therefore do not mature. This question cannot easily be answered but in the next section data will be given which indicate that it might be the pituitary which in this phase is unable to secrete a sufficient amount of gonadotrophins for the maturation of the gonads.

2. RATE OF GROWTH OF YOUNG FISH UNDER DIFFERENT EXTERNAL CONDITIONS

One year it was noticed that under-yearling fish kept at 8 hours day length and 20° C grew faster than those kept under 16 hours and 20° C. This led to the following experiment in which the growth rates were determined in three different groups of young fish subjected to a temperature of 20° and a day length of either 8 or 16 hours.

Experiment 26. On June 14 and July 19, 1954 and on July 11, 1955 groups of young animals that had hatched in the laboratory and were reared in the aquarium-house under almost natural conditions (see fig. 1) were subjected to a temperature of 20° and to a day length of either 8 or 16 hours. The number of fish in each group was the same, while the tanks in which the fish were kept were the same size. The amount and quality of food (abundant live *Daphnia*) was also similar in all groups. The initial average length of the June groups was 3.3 cm (range 2.9–3.6). The corresponding figure for the group of July 11 was 3.0 cm (2.6–3.4) and that for the July 19 group was 2.6 cm (2.4–2.8). The results of this experiment are given in fig. 5.

It can be seen that in each of the three groups the animals exposed

to 8 hours day length and 20° grew much faster than those kept under 16 hours day length and 20°. The dates of maturity of some of the animals in these groups (exposed to 16 hours and 20°) have already been given in fig. 4. In the measurements animals in phase 0 at the beginning of the experiments (those that did not mature) have been included as well as fish in a later phase (phase 1a) that did mature. The result of the above experiment means that animals in phase 0 and

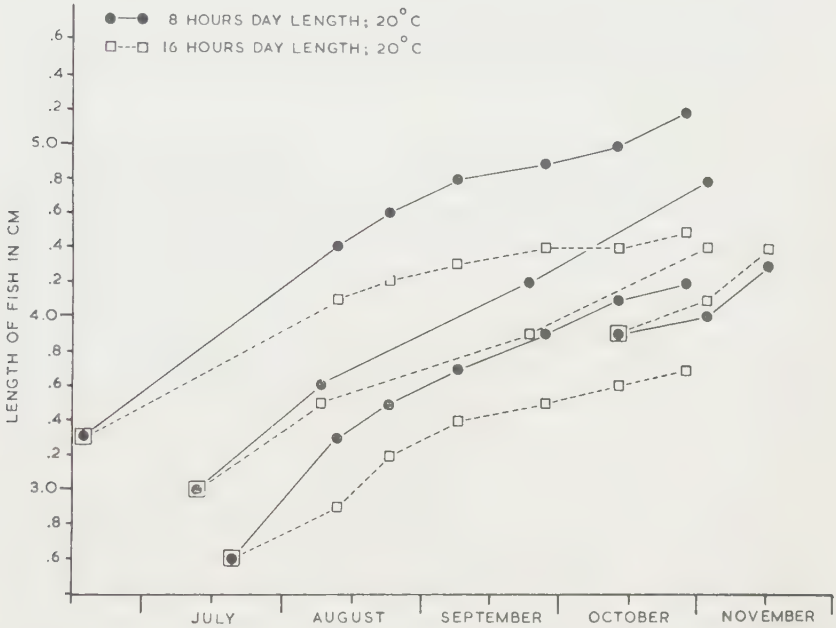

Fig. 5. Experiments 26 and 27. Growth rates of fish (hatched in April and May) of initially the same length exposed at different times of the year to a temperature of 20° C combined with a day length of either 8 or 16 hours.

the early stage of phase 1a grow faster when exposed to 8 hours day length and 20° than when they have been subjected to 16 hours day length and 20°.

Since it has also been shown in Telcosts that the growth hormone secreted by the hypophysis regulates growth (Pickford and Thomson, 1948; Pickford, 1953 a,b) it is suggested that in the above case the production of growth hormone is stimulated much more by 8 hours day length and 20° than by 16 hours and 20°. This would mean that the secretion of growth hormone by the pituitary under the latter condition is far from maximal.

Now the fact that under 16 hours day length and 20° the animals

show neither a fast growth nor gonadal development might indicate that the function of the pituitary in secreting both growth and gonadotrophic hormones is, to a certain extent, inhibited by this particular external condition.

It must be added here that this inhibition only takes place in fishes in gonadal phase 0, for in animals that did attain maturity under these conditions (thus in those which must have been in the very beginning of phase 1a) only the secretion of growth hormone was partially inhibited. In the following experiment it will be shown that in the later part of phase 1a the production of growth hormone is no longer partially inhibited.

Experiment 27. Under-yearling fish that had hatched in April and May 1954 and had been reared in the aquarium-house under almost natural conditions (see fig. 1) were exposed to a temperature of 20° C and a day length of either 8 or 16 hours on October 9. The average length of the fish in both groups was 3.9 cm (range 3.5–4.7) at the beginning of the experiment. From other experiments it is known that the animals at this time were in phase 1 of gonadal development, for they did not mature under 8 hours day length and 20°, but attained maturity when exposed to 16 hours day length and 20°. It is not known in which stage of phase 1 they were, but it is likely that at this time they were either at the end of phase 1a or in phase 1b.

Fig. 5 shows that in the first month the 16-hour group had grown somewhat faster than the 8-hour group while the rate of growth in both groups was the same during the second month. After this time the animals in the 16-hour group matured and the measurements were discontinued. These results indicate that in later stages of gonadal development 16 hours day length and 20° no longer inhibits the secretion of growth hormone.

The conclusion that can be drawn from the experiments described in sections 1 and 2 is that at a very early phase of gonadal development, namely phase 0, 16 hours day length and 20° C partially inhibit growth and completely inhibit maturation. This is most probably caused by an insufficient secretion of both growth hormone and gonadotrophic hormones by the pituitary. It is not known whether a reduced sensitivity of the gonads to gonadotrophins may also play a rôle in the prevention of maturation under these conditions.

The fact that both growth and gonadal development are either retarded or inhibited in very young Sticklebacks under the influence of 16 hours day length and 20° C might play an important rôle in the timing of the breeding season of under-yearling fish. Sticklebacks that hatch in April and May live during late spring and summer under

conditions that are favourable for growth, namely an abundance of food and a high water temperature. If at this time the day length was not about 16 hours, the above conditions would cause a rapid growth of the young animals and also a rapid development of the gonads, with the result that at least some of the animals would spawn about 4 months after hatching (see p. 166), i.e. around August. There is no doubt that this would be an unfavourable time of year for the survival of the offspring. But it has been shown that a long day length combined with a high temperature do inhibit rapid growth and development of gonadal phase 0 to later phases. This means that the above condition prevents an untimely onset of the breeding season in under-yearling animals.

However, the fact that animals in phase 0 do not attain maturity when kept under constant conditions of 16 hours day length and 20° C for over one year (experiment 24), while they do mature in nature in the spring of the next year, implies that in nature there must be an external condition which removes the inhibiting effect on development of phase 0 caused by the long day length and high temperature in summer. The existence of such an external condition has already been demonstrated in fig. 4 where it can be seen that in under-yearling animals that have been kept under almost natural conditions (in the aquarium-house until the end of September) exposure to 16 hours day length and 20° no longer inhibits gonadal development. At this time of the year the day length and temperature are both decreasing and it was therefore supposed that the inhibiting effect might be removed by either a short day length or a low temperature or by a combination of both. Experiments on this subject will be described in the next section.

3. REMOVAL OF THE INHIBITING EFFECT OF 16 HOURS DAY LENGTH COMBINED WITH 20° C ON GONADAL DEVELOPMENT IN VERY YOUNG ANIMALS

In experiment 25 it was shown that when under-yearling fish are first exposed to a short day length and high temperature and subsequently to 16 hours and 20°, the latter condition is no longer able to inhibit maturation. In the following experiments the animals were first exposed to 16 hours day length and 20° C and later to different external conditions in order to make conditions comparable to those in nature, where young animals are also first exposed to a long day length and high temperature, and later to other external conditions.

a. *The effect of a period of 16 hours day length and low temperature*

Experiment 28. The animals used here had hatched in April and May, 1954 and had been reared in the aquarium-house under almost natural conditions (see fig. 1). They came from the same batch as the group of animals that were exposed on July 19 to 16 hours day length and 20° C (see fig. 4). The present group consisted of 5 animals that were also exposed to 16 hours day length and 20° on July 19. On that date they were of the same average length as the animals described in experiment 24. Therefore the latter will be used as controls for the present experiment.

On August 28 the 5 animals were subjected to a period of low temperature, while the day length remained 16 hours. The period of low temperature consisted of a decrease in temperature within 24 days from 17°–4° and a subsequent increase, within 18 days, from 4°–20°. The latter temperature was reached on October 6.

The result was that 4 out of the 5 animals matured before December 15, while the last animal attained maturity on January 29. In figure 4 it can be seen that in the controls (19 July, 1954) none of the animals had matured before December 15, while only one of them had attained maturity by the time all 5 animals of the experimental group had responded (January 29).

It can be concluded that the inhibiting effect of 16 hours day length and 20° C can be abolished by exposing the animals to a period of long day length and low temperature. This means that the latter condition is able to stimulate development of phase 0.

b. *The effect of a period of 8 hours day length and 20° C*

In experiment 25 it was shown that gonads in phase 0 are able to develop when the animals are first exposed to 8 hours day length and 20° and afterwards to 16 hours and 20°. In the following experiment the animals were first subjected to 16 hours day length and 20° and afterwards to 8 hours for comparison with the previous experiment (28).

Experiment 29. The animals used came from the same group as those of July 11, 1955, which were described in experiment 24 (see fig. 4). It can be seen from the figure that when the animals were exposed to 16 hours day length and 20° on July 11, only 6 out of the 17 animals had attained maturity by February 20, 1956. Two of the animals died during this period. On February 20, four out of the remaining nine animals were exposed to a day length of 8 hours and a temperature of 20°, while the rest stayed at 16 hours and 20° and were regarded as controls. On April 4 the experimental fish were transferred back to 16 hours and 20°. During this time one animal in each group had died,

so that only three remained in the experimental and four in the control group.

The result was that all three experimental animals matured within 42 days of the transfer, namely on May 9; 10 and 15. Also two of the controls attained maturity, namely on April 11 and May 22, but the remaining two animals did not mature until August 15 when the experiment was terminated.

This result means that the inhibiting effect of 16 hours day length and 20° C can also be abolished by exposing the animals to a period of 8 hours day length and 20° C. Thus the latter condition is favourable for the development of phase 0. A similar effect of 8 hours day length and 20° has also been shown in experiment 25.

The conclusion from the experiments described in this section is that 8 hours day length and 20° C, as well as 16 hours day length and low temperature, are conditions that are able to abolish the inhibiting effect of 16 hours day length and 20° C on the development of gonadal phase 0. On p. 173 the question was asked what external condition would be able to abolish the inhibiting effect of long day and high temperature on gonadal development to which the young Sticklebacks are exposed in nature after hatching. This question is now answered. The fact that both the day length and the temperature are decreasing during autumn is double assurance that the above inhibiting effect will be removed and that the animals will be able to spawn in the next spring.

It should be mentioned here that phase 0 only occurs in very young fish measuring less than 2.6 cm. It will be shown in Chapter X that adult fish kept for over one year under constant external conditions of 16 hours day length and 20° C show alternative reproductive and non-reproductive periods. Therefore, phase 0 does not occur in the cycle of adult fish.

There is still another conclusion that can be drawn from the results of the experiments described in this section, namely that it is the joint action of 16 hours day length and 20° C which inhibits development of phase 0, and not the action of one of the components alone. For it is known that, *a.* 16 hours and 20° inhibits development, *b.* 8 hours day length and 20° C has no inhibiting effect, and *c.* 16 hours day length and low temperature also does not inhibit development.

This is another example showing that a certain phase of development is affected only by the combination of a particular day length and a particular temperature and not by one of the two components combined with another day length or temperature. The first examples of this kind were given on p. 161, where it was shown that only the specific combination of 8 hours day length and a low temperature is able to

stimulate the development of gonadal phase 1a, while the specific combination of 8 hours day length and 20° C inhibits development of this phase.

4. ACCELERATED MATURATION AND THE APPEARANCE OF THE NUPTIAL DRESS IN MALE STICKLEBACKS

During the breeding season male Sticklebacks are brilliantly coloured. The back and eye have a bluish or greenish shine due to the presence of guanocytes. The area below the mouth, the gill-covers and part of the belly are brilliantly red due to a great number of erythrophores. TITSCHACK (1923), OSTERHAGE (1932), IKEDA (1933), and WUNDER (1934) have given a detailed description of this nuptial dress.

During autumn and winter the red pigment disappears almost completely. The only place where it is still present in most males during winter is on the inside of the bottom of the mouth, where it usually forms a red patch. In females the mouth bottom may sometimes also show a pale reddish shine (most probably due to the presence of blood vessels), but usually this place is partly covered by black pigment. This difference between males and females was always used to select males from females in winter.

In the experiments in which maturation of the gonads was artificially greatly accelerated, e.g. in the under-yearling fish described in section 1 of this chapter and in adult fish caught in mid-winter (see tables 1 and 2) the males at the time of nest-building in most cases showed a very faint nuptial coloration. The eyes were only faintly blue, while a reddish shine was present only around the corners of the mouth and on the gill-covers. This means that the formation of the red pigment could not keep pace with gonadal development. Usually in these cases the full nuptial coloration was ready about 10–25 days after the attainment of maturity. This means that the development of the nuptial dress had also been accelerated, though at a slower rate than gonadal development.

The fact that the process of nuptial coloration lags behind maturation when the latter process is greatly accelerated artificially, is also one of the reasons that CRAIG-BENNETT (1931) using the appearance of the nuptial coloration as a criterion for gonadal development, was unable to demonstrate any effect of day length on maturation in January and later (see Chapter IV). For as can be seen from tables 1 and 2 it is the day length together with the high temperature which, at this time of the year, accelerates maturation much more than the temperature alone. The latter is the reason that CRAIG-BENNETT was able to show an acceleration of maturation by high temperatures by

using the same criterion. For in this case the development of nuptial coloration does not lag so far behind gonadal development.

B FACTORS INFLUENCING THE DURATION AND THE TERMINATION OF THE BREEDING SEASON

The previous chapters have dealt with the problem of the causation of the onset of the breeding condition, but in the following chapters experiments will be described in which an attempt was made to determine the factors which cause the termination of the breeding season. In the literature only very few data are available concerning the latter problem.

In nature the breeding season of the 3-spined Stickleback ends in the second half of June and the first half of July (Leiner, 1930; Craig-Bennett, 1931, and personal observations). At this time the day length shows its annual peak, while the temperature of the water is also almost at its maximum. It is possible, therefore, that these factors, either singly or in co-operation with other external or internal factors, might play a rôle in the termination of the breeding season.

IX. DURATION OF THE REPRODUCTIVE PERIOD UNDER DIFFERENT CONSTANT EXTERNAL CONDITIONS

The following methods were used to determine the end of the reproductive phase in males and females. Male Sticklebacks in phase 2 were induced to mature under different external conditions. After they had built their first nest, they were placed in separate tanks or compartments of tanks, where they could always see other male and female Sticklebacks.

The nests were destroyed at intervals of about 5 days, but the nest-material was left in the tanks or was renewed when necessary. In the majority of cases the nests were rebuilt within a couple of hours and generally within 24 hours. The day on which they did not rebuild their nests was taken as the end of the reproductive period. Some of the males were not allowed to fertilize eggs, while others raised one or more broods.

The females used were also in phase 2 of gonadal development when the experiments started. Care was taken that they got plenty of food, particularly around the time of maturation and during the reproductive phase. When the females were ripe they were either introduced into tanks where males had a nest and where they could deposit their eggs in the nest, or the eggs were stripped (see p. 114).

A very remarkable fact was that when a stripped female was placed in the territory of a male with a nest, she would show courting behaviour and even creep into the nest. Females that have no opportunity to deposit eggs in a nest can also finally get rid of the eggs, but I have never observed how this is done.

When sexually mature females are fed abundantly they can deposit eggs a number of times at intervals of only a few days. The last time they produced eggs was taken as the end of the breeding period.

1. DURATION OF THE BREEDING PERIOD UNDER 16 HOURS DAY LENGTH AND 20° C

a. *Experiments with males*

Experiment 30. A great number of males in phase 2 were used, but in the course of the experiment many of them died. This is the reason that relatively few animals could be kept longer than 4–5 months. The data on the duration of the breeding period are given in fig. 6. In this figure other data, which will be discussed in the following chapter, are also given. In the present chapter we shall restrict ourselves to a discussion of the duration of the first breeding period (black columns on the left). The letters in the margin indicate the individual animals.

It can be seen that most animals have an uninterrupted reproductive phase, but that four, namely A_1, M, Z, and O have interrupted breeding periods, i.e. they started to build nests again after a short period in which they had no nests. These cases will first be left out of consideration.

The average duration of the breeding period in the 24 animals with uninterrupted periods was 91.4 days (range 23–198 days). It is difficult to explain the enormous variations in the duration of the breeding periods. Though the animals used were all in phase 2 of gonadal development at the beginning of the experiment, they nevertheless had been exposed to different external conditions before they reached phase 2; thus some had been subjected to periods of low temperature, others to short day lengths, while again others came from natural habitats. This might have caused the great variation, but it will be shown in section 3, that it is very unlikely that this circumstance played a rôle.

In the second place it is possible that the variation can be explained by the fact that animals that had never spawned before were used, together with others that had spawned at least one or more times before the present experiment started. To find out whether or not

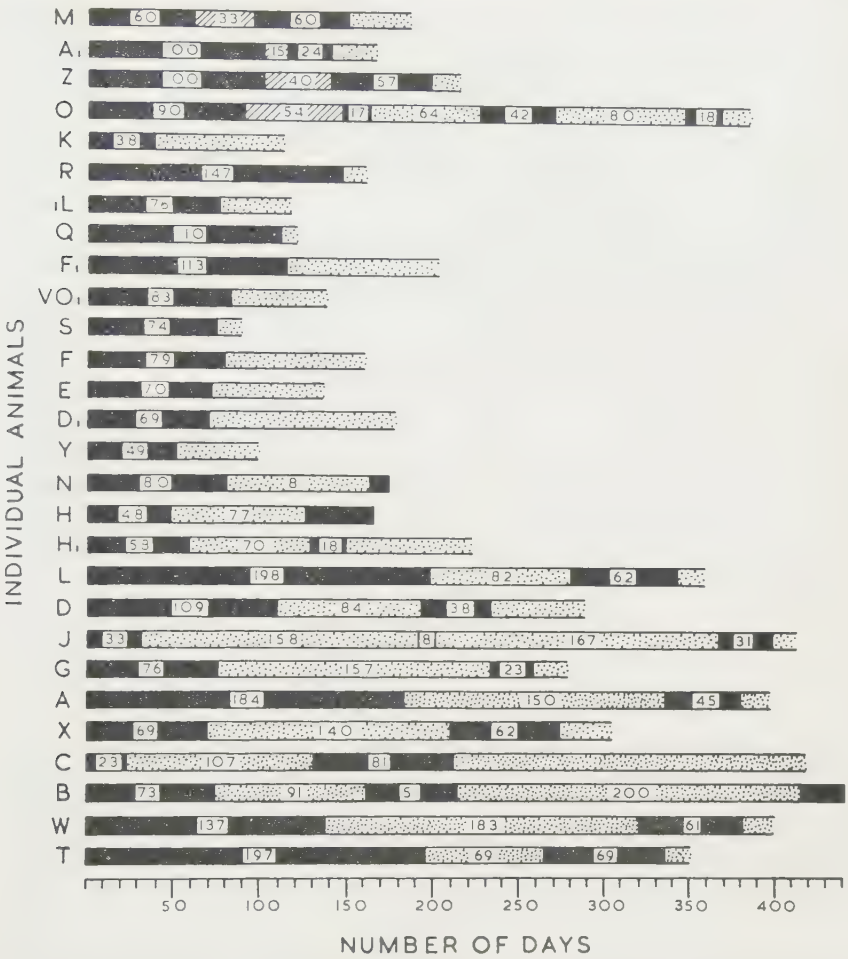

Fig. 6. Experiment 30. Duration of reproductive (black bars) and non-reproductive periods (stippled bars) in animals kept for long periods of time at 16 hours day length and 20° C. Their histories are recorded until about one week before death. Hatched bars: interrupted breeding periods; for explanation see text.

this circumstance may have played a rôle the 24 animals were classified into two groups, one consisting of fish that had never spawned before (young) and one in which the fish had spawned at least once (adults).

The average duration of the breeding periods in the latter group (adults) was 97 days (range 23-198 days), while the corresponding figure for the other group (young) was 77.7 days (range 33–113). There were 17 animals in the first group and 7 in the last. The difference in the

duration of the breeding period in the two groups is not significant. This indicates that the duration of the breeding period might be equally long in young and in adult fish. It is also clear that the great variation in the duration of the reproductive phase that was found above cannot be explained by the fact that both adult and young animals were used, because in each group the scatter is about equally large.

Another circumstance that may have caused the great variation is the fact that some of the animals had been allowed to fertilize eggs and raise young, while others had not. Therefore, the duration of the reproductive phase was calculated for the first and second groups separately. The average duration of the breeding period in the animals that had been allowed to fertilize eggs and raise young (13 animals) was 89.8 days (range 23–198 days), while the corresponding figure for the other group (11 animals) was 93.2 days (range 49–197 days). The difference between the two groups is not significant. It can be concluded that the consummatory act of fertilizing eggs and raising young does not influence the duration of the breeding period under the external condition used in this experiment.

Since no other circumstances are known that could have caused the great variation in the duration of the breeding period of animals kept under constant external conditions, it must be assumed that the differences are caused by individual variations.

The last point that must be discussed here is the fact that in some animals the breeding period is interrupted by a relatively short period in which no nests were built. This was the case in 4 out of the 28 animals (animals A_1, M, Z and O) This interruption is difficult to explain. It is assumed that this period of "rest" is caused by a temporary insufficient production of gonadotrophins by the pituitary or by a temporary insufficient production of gonadal hormones. In the first instance the main cause would lie in the pituitary, in the second instance in the gonads. This temporary insufficient function of either or of both organs, would then be followed by a period in which the production of hormones again becomes sufficient to keep the animals in the nestbuilding and thus in the reproductive phase. In this case it is assumed that during the period of "rest" no total reorganisation of the gonads takes place as happens after the end of the breeding period. This assumption is supported by the following facts.

The duration of the period of "rest" in the four animals was 15, 33, 40 and 54 days respectively; mean duration 35.5 days. In the following chapter it will be shown that the mean duration of the interval between two reproductive periods under 16 hours day length and 20° C is 108 days (range 64–158 days). The difference between the duration of the "rest-period" and the latter period is significant ($P = 0.002$). This means that the duration of the rest-period it too short for testis reorganisation and can therefore not be compared with the interval between two reproductive periods.

Another fact that supports the above assumption is that during the period of "rest" the animals retained their nuptial dress, though its brightness was temporarily subdued by melanophores covering the erythrophores. As soon as the animals

started to build nests again the brilliant colours reappeared. During the interval between two reproductive periods the nuptial coloration disappears completely.

In the four animals the duration of the first part of the breeding period was 100, 60, 100, and 90 days, mean duration 87.7 days. The total duration of the breeding period, excluding the period of "rest" was 124, 120, 157, and 107 days respectively, mean duration 127 days. The total duration of the breeding period including the "rest"-period was 139, 153, 197, and 161 days respectively, mean duration 162.5 days. When these mean durations (87.7, 127, and 162.5 days respectively) are compared with the duration of the breeding period in animals that have an uninterrupted reproductive period, namely 91.4 days (range 23–198 days) it seems as though the last two figures for the above four animals are larger, though the difference is statistically not significant. However, more data are needed before a definite conclusion can be drawn on this point.

b. *Experiments with females*

Experiment 31. In this experiment only under-yearling fish of about 5.1 cm were used. From an experiment made with animals of the same group that were exposed to 8 hours day length and 20° C it is known that at the beginning of the experiment (in November) the animals had reached phase 2. They were maintained under 16 hours day length and 20° C for more than two months following maturation. A total number of 12 animals was used, but 3 died in the course of the experiment (the data on the latter fish were discarded).

The result of this experiment was that the (9) females deposited eggs on the average 10.8 times (range 4–15 times). The number of days passing between two egg-depositions was on the average 6.2 (range 3–16), while the total duration of the reproductive period (measured from the first until the last oviposition) was on the average 60 days (range 31–77 days). The eggs at the end of the reproductive period matured as fast as the eggs at the beginning of this period, for the mean number of days between the first four ovipositions was 6.7 (range 4–10), while the corresponding figure for the last four spawnings was 5.9 (range 3–16 days). This difference is not significant. It seemed, however, that the number of eggs produced in the last ovipositions was smaller than in the first, though no quantitative data on this subject are available.

This experiment shows that female Sticklebacks can spawn as many as 15 times in one season, provided that abundant food is present. The external conditions during this experiment are not so much different from conditions in nature between April and July, for at this time the day length increases from 14–16 hours, while the temperature is also rather high (see fig. 1).

The experiment was made with under-yearling fish and it might be possible that adult females spawn more often and during a longer period of time. The spawnings of one adult female under the same

conditions as the above young are known. This animal deposited eggs 8 times, with an interval of 5–10 days, during a period of 45 days. This result does not indicate that there would be a difference between the number of spawnings of young and adult females, but more data are needed before a definite conclusion can be drawn.

The first conclusion that can be made at the end of this section is that in males as well as females there must be an intrinsic factor which terminates the breeding period under the constant external condition of 16 hours day length and 20° C. The action of the internal factor is such that under constant external conditions breeding is possible only during a restricted period of time. We shall return to this point in more detail on p. 186.

In the second place it can be concluded that the duration of the breeding period in the males lasts longer than in the females (91.4 and 60 days respectively). The difference is significant at the 0.05 level. The variations in the duration of individual breeding periods is smaller in the females than in the males (31–77 and 23–198 respectively).

In the third place it has been shown that the duration of the breeding period is most probably equally long in "young" and "adult" males, while fertilizing eggs and raising young does not influence the duration.

2. DURATION OF THE BREEDING PERIOD UNDER 8 HOURS DAY LENGTH AND 20° C

a. *Experiments with males*

Experiment 32. All animals used were in phase 2 of gonadal development at the beginning of the experiment, for they matured under the stimulus of 8 hours day length and 20° C. The results of this experiment are given in fig. 7.

The data show that the mean duration of the reproductive period of 32 animals (with uninterrupted breeding phase) was 45.8 days (range 5–112). As in the experiment with 16 hours day length and 20° C the duration of the reproductive periods in the individual animals is considerably different.

This variation might be caused by the different conditions the animals had been exposed to before they reached phase 2. In the following section, however, evidence will be given which indicates that it is not very likely that this circumstance could have played a rôle, though this evidence is not fully conclusive.

In the experiment both "young" animals (fish that have never spawned before) and "adults" (animals that have spawned at least

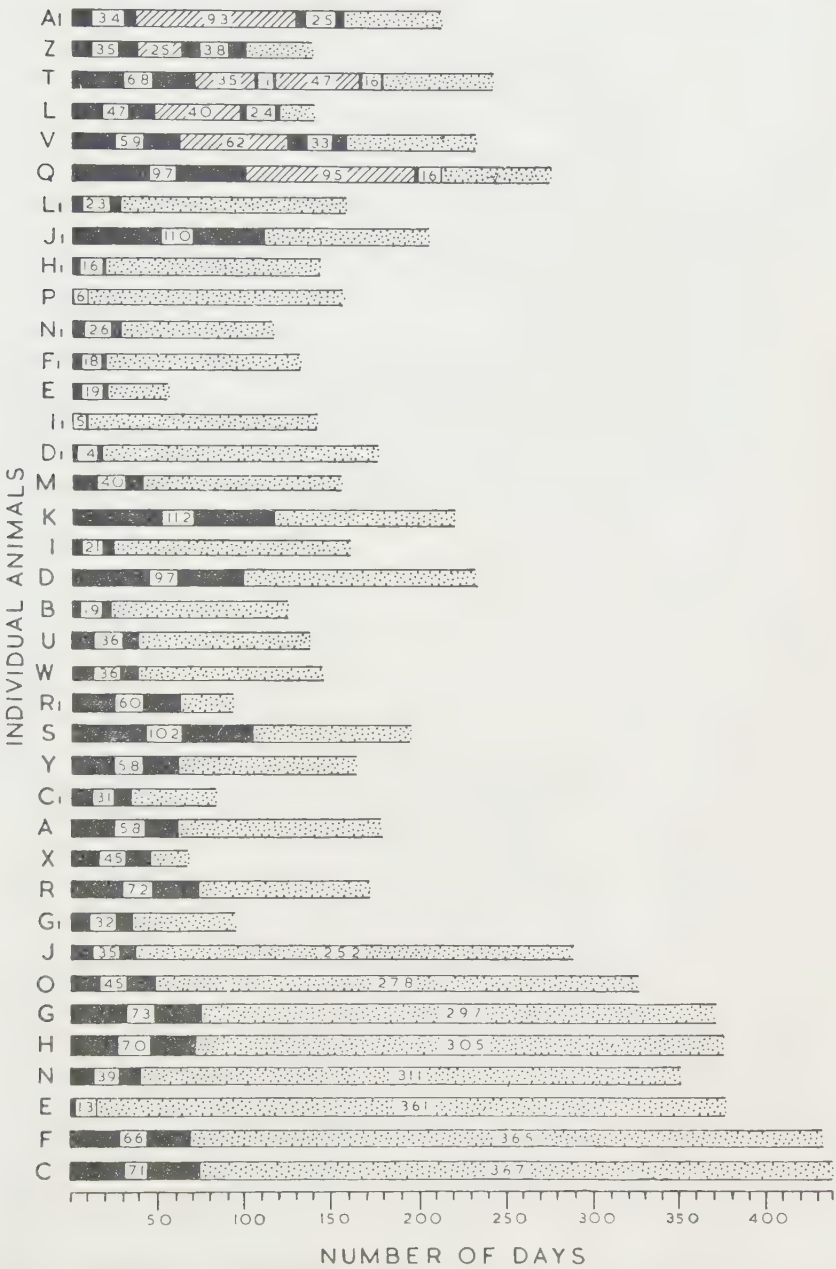

Fig. 7. Experiment 32. Duration of reproductive (black bars) and non-reproductive periods (stippled bars) of animals maintained for long periods of time at 8 hours day length and 20° C. For further explanation see fig. 6.

once before) have been used and this might have given rise to the great variation in the duration of the breeding phase. The mean duration of the reproductive period in the 21 adult fish was 48.3 days (range 5–102), while the corresponding figure for the young animals was 41.2 days (range 6–112). The difference is not significant. This result means in the first place that the duration of the reproductive phase lasts equally long in both "adult" and "young" fish under the above condition. In the second place the great variation present in each group means that the above-mentioned variation in the duration of the breeding phase cannot have been due to the fact that the group consisted of both adult and young fish.

A third possibility to explain the variation might be the fact that some animals had been allowed to fertilize eggs and raise young while others had not. The average duration of the breeding phase in the former group (which had raised young) was 59.6 days (range 26–112), while in the latter group the corresponding figure was 42.1 days (range 5–110). This difference is almost significant at the 0.05 level. Unfortunately the number of fish that had raised young was rather small (8) in comparison with the number of animals that had not raised young (24). Thus more data are needed before a definite conclusion can be drawn. But the difference indicates that there might be a tendency in animals to breed longer under 8 hours day length and 20° C when they have fertilized eggs and raised young than when they have not. Such a difference could not be found in animals kept under 16 hours day length and 20° C. However, the fact that again in both groups the individual variations in the duration of the breeding period are very great cannot have been the cause of the great variation found above. It seems, therefore, that this variation is caused by individual differences with respect to the duration of the breeding period.

It can be seen from fig. 7 that, as with the animals kept under 16 hours and 20° C, there are also some animals here which show interrupted breeding periods (animals A_1, Z, T, L, V, and Q). It is assumed that also in this case the period in which the animals did not build must be regarded as a period of insufficient functioning of either the pituitary or the gonads or of both (period of "rest", see p. 180). During this period no testis reorganisation takes place as in the interval between two reproductive periods. This assumption is supported by the fact that it is known that animals in phase 1 (i.e. one of the phases of gonadal development that must be passed during gonad recrudescence) are not able to mature under 8 hours day length and 20° C (see Chapters VI and VII). The animals also did not loose their nuptial coloration during the period of "rest", while they do during the non-reproductive period.

In the above 6 animals the mean duration of the period until "rest" was 56.7 days (range 34–97). The average duration of the rest-period was 59.3 days (range 25–95) while the mean duration of the second nest-building period was 24.5 days (range 11–38). One animal (T) even showed a second period of rest (47 days) followed

by another nest-building phase lasting 16 days. The total duration of the breeding period in these 6 animals, excluding the periods of rest, was 85 days (range 59–113), while the total duration of the breeding period including the rest-periods was on the average 143.1 days (range 98–208).

When these data are compared with the duration of the breeding periods in animals that showed no interruption of the lat er period namely, mean duration 45.8 days (range 5–112), it can be calculated that the difference between the latter duration and that of the first nest-building period (until rest) is not significant, but that there is a significant difference between the former figure and that of the total duration of the breeding period, both when the rest-period is excluded as well as included. This means that during the period of rest some recovery of the pituitary or gonad function takes place enabling the animals to continue breeding during a longer period than fish in which these functions did not recover.

When the total duration of the breeding period including the period of rest (143 days) is compared to the corresponding duration of the breeding phase in animals kept under 16 hours day length and 20° C (162 days) there seems to be a difference in favour of the latter fish, though this difference is not significant. The result indicates that in a few cases the duration of the breeding phase under 8 hours day length and 20° C may last about as long as that under 16 hours day length and 20° C, though in the majority of cases the latter lasts much longer.

b. *Experiments with females*

Experiment 33. Fourteen young females were used that were all in phase 2 at the beginning of the experiment, for they responded to the day length of 8 hours combined with a temperature of 20° C. Treatment before exposure to the latter condition was the same in all animals.

The result of this experiment was that the mean number of ovipositions was only 2 (range 1–3). The average number of days between two ovipositions was 5.9 (range 4–8). The mean duration of the total breeding period was 6.6 days (range 4–13).

When these figures are compared with those obtained in animals kept under 16 hours day length and 20° C (see p. 181) it can be seen that there is a considerable difference between the number of spawnings and the total duration of the breeding period (both differences are significant at the 0.01 level). There is no difference between the duration of the intervals between two ovipositions.

It is not known whether adult females would have remained longer in breeding condition, but it does not seems very likely (see also p. 181).

This result means that the breeding season in the females is also shorter under 8 hours day length and 20° than under 16 hours and 20°.

The conclusion that can be made at the end of this section is that under the constant external condition of 8 hours day length and 20° C there must also be an intrinsic factor which terminates the breeding season. However, breeding lasts longer under 16 hours day length and 20° (males 91.4, females 60 days) than under 8 hours day length and

20° (males 45.8, females 6.6 days). This means that the intrinsic factor that terminates the reproductive phase under the two constant conditions mentioned above acts in co-operation with external factors. When there is a strong external stimulus the intrinsic factor takes longer to terminate the breeding period than when the external stimulus is weaker. Therefore, I assume that the intrinsic mechanism responsible for breeding needs a certain amount of external stimulation for maintenance of this condition. As soon as the ability of the internal mechanism to maintain breeding becomes exhausted the breeding period is ended. The ability of the internal mechanism to maintain breeding might vary much in the individual animals and this would explain the great variation in the duration of the breeding period. For in this way it is possible that under the same external condition some animals breed only for a short time, while others may breed for three or more months.

Other conclusions that can be drawn are that the duration of the breeding season in the males (45.8 days) lasts significantly longer than in the females. Animals that have never spawned before breed about as long as those that have spawned at least once. There is a tendency for males that have fertilized eggs and raised young to breed longer under 8 hours and 20° C than those that have not. It seems that the fertilization and raising of young has stimulated the internal mechanism responsible for the maintenance of breeding. This effect was not seen in the corresponding animals kept under 16 hours day length and 20° C, most probably because the strong stimulus of the external condition overshadowed the much weaker stimulus of fertilization and raising of young.

3. THE INFLUENCE OF EXTERNAL CONDITIONS PRIOR TO EXPOSURE TO 8 HOURS DAY LENGTH AND 20° C ON THE DURATION OF THE BREEDING PERIOD UNDER THE LATTER CONDITION

It has been mentioned in sections 1 and 2 that, though the animals used were in the same gonadal phase at the beginning of the experiments, they had been exposed to different external conditions before this phase was reached. It was also mentioned that this fact might have been the cause of the great variation in the duration of the breeding condition in the various animals. This consideration led to the following experiment.

Young animals were used that had been exposed to 16 hours day length and 20° C in phase 1 and had subsequently matured under

the same condition. The males and females were transferred to 8 hours day length and 20° C on the day on which they built their first nests, or had deposited eggs for the first time. They remained under the latter condition until the end of the breeding period.

a. *Experiments with males*

Experiment 34. Six under-yearling animals were used. The average duration of the breeding period in these fishes was 57 days (range 11–169). The duration of the breeding condition in the animal that bred 169 days might even have been longer, because it died one night when the water was overheated due to a failure of the thermostate. These figures compared with those obtained with animals that had been exposed to 8 hours day length and 20° C right from the beginning of the experiment, namely 45.8 (range 5–112), show that there is a tendency for the former to breed longer, though the difference between the responses of both groups is not significant. Thus in male Sticklebacks the duration of the breeding season under 8 hours day length and 20° C does not seem to be affected by a previous exposure to 16 hours day length and 20° C. However, the number of animals that was used was rather small and more data are needed before a definite conclusion can be drawn. It is also shown that in these animals, which had had the same treatment before the experiment, there is again a considerable variation in the duration of the breeding period. Therefore, it is unlikely that the great individual differences shown in the previous experiments were due to exposure to different external conditions prior to the experimental condition. However, to be quite sure about this point one should also test the influence of various other external conditions.

It must be added here that the above conclusion only holds for males, for it will be shown in the following experiment that females react differently.

b. *Experiments with females*

Experiment 35. In this case 12 young females were used. The mean number of spawnings in this group was 3 (range 1–5), while the duration of the breeding period was 11.1 days (range 9–18). The number of days between two ovipositions was 5.4 (range 3–11). The corresponding figures for the animals that had been exposed to 8 hours day length and 20° C from the beginning of the experiment are 2(1–3), 6.6(4–13) and 5.9(4–8). The difference between the duration of the breeding periods in the two groups is significant at the 0.005 level. This means that the duration of the breeding period in females that have first been exposed to 16 hours day length and 20° C and subsequently to 8 hours

day length and 20° C is longer than that of animals that have been exposed to the latter condition right from the beginning of the experiment. However, the duration of the breeding period in the former animals is still shorter than that of animals exposed to 16 hours day length and 20° C from the beginning of the experiment (60; range 31–77).

It is difficult to explain the difference in response to the above conditions between the males and the females. In the case of the males the previous exposure to 16 hours day length and 20° C did not significantly affect the duration of the breeding period under 8 hours and 20°, though it can be seen that in these animals there is also a tendency for the breeding period to become longer. Perhaps when more data are available on the response of the males more definite conclusions can be drawn.

Unfortunately no experiments could be made with other external conditions.

The result obtained with females indicate that the internal mechanism maintaining breeding can also be favourably influenced by the stimulus of an external condition to which the animals had been exposed prior to the onset of breeding. Though the data on the same subject in males are not conclusive, the latter show a similar tendency as the females. It seems as if conditions prior to the attainment of maturity have a kind of "after-effect" on the maintenance of breeding. Again it is clear that the reproductive phase has been ended by the co-operation between external and internal factors. Under the present conditions the males again breed longer than females.

4. DISCUSSION

First a short summary will be given of the results obtained in the experiments described in this chapter. To make this easier, the external condition of 16 hours day length and 20° C will be called "condition 1". The condition of 8 hours day length and 20° C will be called "condition 2", and that of 16 hours and 20°, followed by 8 hours and 20° will be called "condition 3".

Under all three conditions the duration of the breeding period is determined by a co-operation between external stimuli and an intrinsic factor (s). The fact that breeding ends after a certain period of time indicates that the fish have entered a refractory period in which neither external or internal factors can stimulate them to breed.

The duration of the breeding period is longer in the males than in the females under all three conditions. There is no difference between the duration of the reproductive phase in "young" and "adult"

animals (at least in the males), under conditions 1 and 2. Under condition 1 there is no difference between the duration of the breeding period in males that have fertilized eggs and raised young and in males that have not. However, under condition 2 there is a tendency in the former group of males to breed longer than those of the latter group.

With regard to the termination of the breeding season in nature the following can be said. The breeding season of the 3-spined Stickleback ends in the second half of June and the beginning of July. At this time the day length is 16 hours, while the temperature is rather high, namely about 18° to 20° C. This means that in nature the breeding season will also be terminated by the co-operation between intrinsic and external factors, as was shown in section 1. The fact that the external condition of 8 hours day length and 20° C is able to induce a premature end of the breeding period might only play a rôle in autumn in the case of animals that would still be breeding at that time. It seems, however, very unlikely that this factor would play an important rôle in nature (see also following paragraph).

The duration of the breeding season in nature lasts about 2.5–3 months. This is in full agreement with the fact that under 16 hours day length and 20° C the average duration of the breeding phase is about 91 days. The latter condition is almost comparable to the natural conditions between the middle of April and the middle of July, particularly with respect to the day length. With regard to the very long breeding periods of some animals kept under 16 hours day length and 20° C (namely 5–6 months) it is possible that such extreme cases are excluded by the fact that day lengths in nature are decreasing in the months after July (effect of 8 hours day length). Condition 3 might play a rôle by increasing the duration of the reproductive phase in summer when the day lengths become shorter, but it does not seem very likely. The fact that the female is able to spawn several times within one season is of course favourable for the survival of the species.

Statements of Wunder (1928) that female Sticklebacks spawn only once during the breeding period, and of Leiner (1930) that this would happen about 3 times, are incorrect and are probably due to the fact that the external conditions under which they kept their fish were not optimal.

In fishes no more data are available on the subject of the termination of the breeding period, but in birds some experiments have been done on this subject (Clark *et al.*, 1937; Burger, 1947, and Wolfson, 1952) have shown that under constant external conditions (long day lengths) the gonads show spontaneous regression after some time. This can only be explained by assuming that an intrinsic factor must have ended the breeding period.

In addition Wolfson (1952) found that long day lengths resulted in an earlier termination of the breeding phase than short day lengths. This means that also in birds external factors are involved in the termination of breeding. However, this finding in birds is opposite to the results obtained in Sticklebacks. Marshall (1955) mentions the fact that "unfavourable" external conditions are able to induce an untimely termination of breeding.

X. THE OCCURRENCE OF ALTERNATIVE REPRODUCTIVE AND NON-REPRODUCTIVE PERIODS UNDER CERTAIN CONSTANT EXTERNAL CONDITIONS

1. CYCLE OF REPRODUCTIVE AND NON-REPRODUCTIVE PERIODS UNDER 16 HOURS DAY LENGTH AND 20° C

a. *Experiments with males*

Experiment 36. The animals that had been used in experiment 30 to determine the duration of their breeding period were maintained after the end of the breeding phase under the same external condition, namely 16 hours day length and 20° C. After a certain period these animals started to build nests again. This is shown in fig. 6. During the experiment a number of animals died, but 14 animals started a second breeding period. In these animals the average duration of the first non-reproductive phase was 108 days (range 64–183 days). Two of the fish died during the second reproductive phase, but the remaining 12 survived. The mean duration of the second breeding period was 46.7 days (range 8–81 days). In the course of the second non-reproductive phase many more fish died and only 3 started a third breeding period. The mean duration of the second non-reproductive phase was 149 days (80, 167 and 200 days respectively). One of the remaining animals died in the course of its third breeding phase, but 2 survived and the duration of their reproductive phase was 18 and 31 days respectively (mean duration 25 days). The refractory periods (duration unknown) are included in the non-reproductive phases.

The figures show that under constant external conditions of 16 hours day length and 20° C the animals show a cycle of alternative reproductive and non-reproductive periods. In the second place it is evident that the duration of the reproductive periods becomes shorter in the course of time, while the duration of the non-reproductive phases becomes longer. From the figures given above it can be seen that the total duration of the first reproductive plus the first non-reproductive period (91 + 108 = 199 days) is about as long as the total duration of the second reproductive plus the second non-reproduc-

tive period (47 + 149 = 196 days). This indicates that under the above conditions the animals have a 200-day reproductive rhythm.

This rhythm is caused by two different factors, the cooperative action of an intrinsic factor together with external factors which ends the breeding period, and the external condition of 16 hours day length and 20° C, which initiates breeding, for it has been shown on p. 153 that in the absence of certain external conditions there is no intrinsic factor which initiates breeding.

The fact that the duration of the breeding periods becomes shorter in favour of the non-reproductive phases might be explained in several ways. It is possible that the long time under the same external condition has affected the gonads or the whole physiological condition of the animals in an unfavourable way. It could also be suggested that the animals have become older and are therefore no longer able to breed for such long periods of time. However, this suggestion seems to be refuted by the fact that under the external condition used here there was no difference between the duration of the breeding periods in "adult" and "young" fish.

The present experiment shows that adult fish have no phase 0 of gonadal development, for it has been demonstrated in Chapter VIII that this phase cannot be stimulated to develop further under the influence of 16 hours day length and 20° C. In the experiment described here the animals, though maintained under the above condition for long periods of time, showed alternative reproductive and non-reproductive periods.

b. *Experiments with females*

Experiment 37. In this case four females were used that were in phase 2 of gonadal development when they were exposed to 16 hours day length and 20° C. They matured around the end of March. The exact dates of oviposition are not known, nor the number of spawnings. Assuming, however, that the mean duration of the reproductive phase is 60 days (see p. 181) this would mean that the end of the reproductive period in these animals was reached around the end of May. The dates of the first ovipositions of the second breeding period were on 3, 8, and 23 of November, i.e. about 160, 165, and 180 days had passed since the end of the breeding period. One animal failed to mature. The result indicates that the average duration of the non-reproductive period was about 168 days. It was impossible to determine the duration of the second breeding period, because at that time there was no live food available which is necessary for regular oviposition.

The data show that also in the females under the above external condition there is an alternation of reproductive and non-reproductive

periods. The total duration of the first reproductive and the first non-reproductive period is approximately 60 + 168 = 228 days. This indicates that the reproductive rhythm of the females under the condition of 16 hours day length and 20° C is also one of about 200 days like that of the males (see p. 191). In the males the reproductive phase is longer than in the females, but in the latter the non-reproductive phase is longer.

The cycle in the female can be explained in the same way as in the male; an internal factor together with external factors end the breeding period and the new breeding phase is initiated by the external condition of 16 hours day length and 20° C. Adult females also no longer have any phase 0 of gonadal development; otherwise it would have been impossible for them to show the cycle of reproductive and non-reproductive periods under the above condition.

2. THE ABSENCE OF A CYCLE OF REPRODUCTIVE AND NON-REPRODUCTIVE PERIODS UNDER 8 HOURS DAY LENGTH AND 20° C

This experiment was only done with males, but since previous experiments have never demonstrated any essential difference[1] between the responses of males and females to the same external conditions, it can be safely assumed that in the present case the females would also have responded in the same way as the males.

Experiment 38. In this case the animals used in experiment 32 were left under the same condition for long periods of time. Fig. 7 gives the results. It can be seen that none of the animals attained maturity, even when some of them were kept for over one year under the same condition. This result is in perfect agreement with that obtained in the experiments of Chapter VI, in which animals were exposed to the above condition in phase 1 of gonadal development. In the present experiment males were kept under the same external condition for the following periods of time without maturing: 367, 365, 361, 311, 305, 297, 278, and 252 days. Most of the animals died in the course of the experiment, but one animal was transferred to 16 hours day length and 20° C after a stay of 365 days under the above condition. It matured 20 days after the transfer.

The result of the present experiment shows that in this case no alternation of reproductive and non-reproductive periods could be

[1] By essential difference is meant that one sex would respond and the other not.

induced, because the external condition inhibited maturation. Again it is shown that under the above condition there is no intrinsic factor initiating the onset of reproduction.

3. DISCUSSION

It has been shown in several chapters that the breeding season is induced by the action of certain external conditions and that no intrinsic factor is present which is able to induce maturation in the absence of these external factors. It has also been shown that under 16 hours day length and 20° C external factors together with an intrinsic factor terminate the breeding season. The experiment described in section 1 of this chapter gave definite proof for this timing-mechanism which is responsible for the occurrence of the annual breeding season. The duration of the (annual) rhythm in nature and of that under 16 hours day length and 20° C is of course different, because the external factors are different. But the main fact here is that the rhythm is caused by the co-operation between external and intrinsic factors.

In birds, which are the only animals so far in which this subject has been studied, it has been found that a second breeding period cannot be induced when the animals are subjected to long day lengths after the end of the first breeding phase (see p. 189). In these animals a new breeding period can only be induced when they have been subjected to a period of short day length following gonadal regression (BURGER, 1947 and WOLFSON, 1952).

XI. THE PHYSIOLOGICAL MECHANISM OF THE TIMING OF THE BREEDING SEASON BY EXTERNAL AND INTRINSIC FACTORS

In this chapter a very brief description will first be given of the interrelationship that generally exists between the pituitary and the gonads and of the influence of gonadal and gonadotrophic hormones on reproductive behaviour. Then a few data will be given which show that a similar pituitary–gonad relationship exists in the 3-spined Stickleback. In a following section the way in which external and internal factors generally affect the breeding cycle will be discussed, while the experiments made with the 3-spined Stickleback will be regarded in view of the general concept on the pituitary-gonad mechanism.

1. THE PITUITARY-GONAD RELATIONSHIP

It is a well known fact that gonadal maturation depends on the presence in the blood of gonadotrophic hormones secreted by the pituitary (the influence of other hormones, e.g. thyroid and adrenal hormones, will be left out of discussion here). Hypophysectomized animals show gonadal atrophy, but this effect can be counteracted by the administration of gonadotrophins. The gonads produce gonadal hormones that are responsible for development of the secondary sexual characters and for at least part of the reproductive behaviour. Gonadotrophic hormones are not thought to have a direct effect on behaviour, but in recent years it has been shown that they may induce part of the spawning behaviour in the fish *Fundulus* (see below). There is a delicate interrelationship between the activity of the pituitary and that of the gonads. A possible excess (or deficiency) in the secretion of gonadotrophic or gonadal hormones is counteracted by a decrease (or increase respectively) in the secretion of gonadal or gonadotrophic hormones. Literature on this subject can be found in all physiological and endocrinological handbooks, while the book of Beach (1948) gives a wealth of information on the subject of the influence of gonadal hormones on behaviour.

The above concept is mainly based on studies in mammals but there is already much evidence that the same concept can be applied to the majority of Vertebrates. In fishes, however, investigations on his subject have been relatively scanty. The available data have recently been reviewed by Hoar (1951b, 1955) and by Dodd (1955), and the reader is referred to these reviews for further references. The present view is that a reciprocal relationship between the activity of the pituitary and the gonads also exists in fishes. Hypophysectomy causes gonadal atrophy and gonadotrophic hormones are able to induce gonadal development and maturation. The gonadal hormones are responsible for at least part of the reproductive behaviour, while it has been shown in recent years that gonadotrophic hormones are responsible for the induction of part of the spawning behaviour in *Fundulus* (Pickford, 1952; Wilhelmi, Pickford, and Sawyer, 1955).

2. EVIDENCE FOR THE EXISTENCE OF THE PITUITARY-GONAD RELATIONSHIP AND ITS INFLUENCE ON REPRODUCTIVE BEHAVIOUR IN THE THREE-SPINED STICKLEBACK

a. *Castration experiments*

Castration has been carried out in the 3-spined Stickleback by Bock (1928a), Ikeda (1933), Craig-Bennett (1931), and the author of the present paper (see also Part II). All investigators found that in

the male the secondary sex characters (e.g. the red belly and the blue eye) gradually disappeared after the operation. Experiments of the author showed that no sign of nuptial coloration returned in fully castrated males that were held in a healthy condition for over 8 months before they had to be sacrificed. The colour of the castrated males is a greenish brown and they cannot be distinguished from males caught in nature in early winter. But it was also observed that the slightest remnant of testis-tissue that remained in the animals after the operation ultimately caused the revival of the secondary sex characters and reproductive behaviour. It was noticed that in these cases a tiny piece of testis-tissue, not more than 0.5 mm in diameter, was sufficient to cause full nestbuilding and courting behaviour.

With regard to the effect of castration on behaviour the author's experiments showed that males gonadectomized in the sexual phase (see p. 113) very soon ceased to perform sexual activities, though usually some aggressive behaviour remained. VAN IERSEL and BURGGRAAF (pers. comm.) castrated males in the sexual phase and found that when given eggs they started a normal parental cycle, though at a somewhat lower level. It shows that the gonads are not essential for parental behaviour. This was confirmed by personal experiments. The result might be an indication that parental behaviour is induced and maintained by prolactin secreted by the pituitary.

b. *Experiments with hormones*

VAN IERSEL (pers. comm.) administered testosteron-proprionate to male Sticklebacks in winter condition. He found that this hormone considerably increased the aggressive, nestbuilding and sexual activities as compared with the behaviour of non-treated males kept under the same external conditions.

CRAIG-BENNETT (1931) injected mammalian A.L.P. extract into fish previously irradiated by X-rays and found that it caused a redevelopment of the interstitial tissue. The author's experiments with mammalian gonadotrophins showed that these hormones increased sexual activities, most probably by the mediation of gonadal hormones. One of the experiments will be described below.

Experiment 39. The preparation used was "Pregnyl"[1] made from pregnancy urine, that mainly contains the I.C.S.H. component, but also very little F.S.H. The preparation can easily be dissolved in water and since it was very difficult to inject Sticklebacks it was decided to let the animals swim in a solution of the hormone.

[1] The author wishes to express her gratitude to the Directors of Organon Ltd. (Oss) for kindly supplying her with the gonadotrophic preparations "Pregnyl" and "Gestyl".

The experiment was carried out in the following way. The experimental as well as the control fish were kept in large aquaria, but were daily placed for 3–5 hours in glass jars containing one liter of water. In the case of the experimental fish increasing amounts of hormones were dissolved in the water. The water of both control and experimental animals was renewed each day.

The animals used were under-yearling fish that had hatched in the laboratory and had afterwards been reared in the aquarium-house under almost natural conditions (see fig. 1, 1952). On January 8 they were transferred to a heated room. Unfortunately the temperature could not be kept constant and was always lower in the morning than in the afternoon. During the experiment it varied between 12° and 17.4° C. The day length to which the animals were exposed was the natural day length at that time of the year (see fig. 1) plus one hour of artificial light of about 25 f.c. The average length of the four experimental and four control fish was 5.2 cm (range 4.6–5.7 cm). At the beginning of the experiment they were presumably in phase 2 of gonadal development.

The experiment was carried out for two reasons. In the first place it would be interesting to know whether gonadotrophins could accelerate maturation and in the second place information could be obtained on the influence of the hormones on reproductive behaviour.

To determine the effect on behaviour daily records were made of some of the reproductive activities, namely aggressive, nestbuilding, and courting activities (see Van Iersel, 1953). The aggressive activities that were distinguished are biting, charging followed by biting, and spine-fights. The nestbuilding movements recorded were sand-digging, searching for material, bringing material, testing, glueing, pushing, sucking, boring, and bringing sand. The only courting activity performed by the males under these conditions was the zig-zag dance. In a male in which the gonads are developing aggressive activities (concerned with the defending of the territory) precede the nestbuilding activities, but the former movements can also be carried out during the nestbuilding and sexual phase (see Van Iersel, 1953). The behaviour of one male in the experimental and one male in the control fish was followed from the beginning of the experiment. Both were the most active fish in their group.

The result of this experiment is given in fig. 8. In the figure the total number of aggressive activities and the total number of nestbuilding plus courting activities are plotted for periods of 30 minutes daily. On some days the animals were observed both before and 1–2 hours after the hormone-bath. In the same figure the amount of hormones, the duration of the hormone-bath and the temperature at the time of observation are also given.

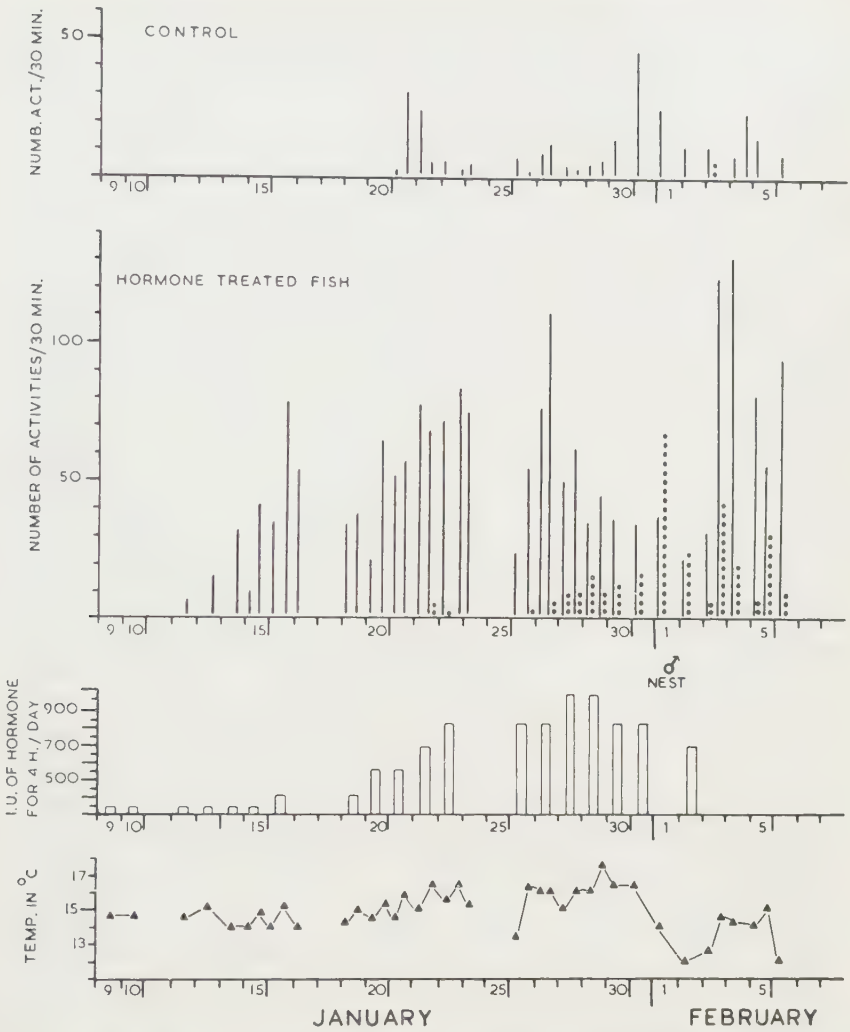

Fig. 8. Experiment 39. The influence of mammalian gonadotrophic hormone on aggressive (drawn lines) and sexual activities (broken lines).

In the first place it is evident that there is a great difference between the behaviour of the experimental and the control fish. In the former the aggressive activities start much earlier than in the latter, while in the control the total number of the activities is always fewer than in the experimental fish during the same period of time. Nest-building movements also start earlier in the hormone treated fish than in the controls. Finally it appeared that the average date of maturity in the

hormone-treated animals was February 3, i.e. 25 days after the beginning of the experiment (range 20–35), while the corresponding figure for the controls was 50 days (range 36–68). This difference is significant ($P = 0.029$).

All these observations indicate that the hormone is taken up by the animals and influences the development of the gonads in that it accelerates maturation and increases the production of gonadal hormones. The latter conclusion is based on the fact that in the majority of cases the number of activities carried out after the hormone treatment is larger than before the treatment on the same day. This fact can only partly be accounted for by the higher temperatures at the end of the day, for in the controls, which experience a similar rise in temperature, the number of activities at the end of the day is only in a very few instances higher than at the beginning of the day.

It can also be seen that during the first weeks of treatment the reproductive activities depended on the presence of a sufficient amount of hormone, for the number of activities decreased as soon as the hormone treatment was stopped. But after the animal had attained maturity its activities were no longer dependent on the presence of the extra gonadotrophins. The decline in the amount of aggressive activities around February 1, when the nest was built, must be explained by the fact that the animal had to spend part of its time on other activities, viz. nestbuilding, so that less time was available for aggressive activities.

This experiment clearly shows that (mammalian) gonadotrophins are able to accelerate maturation and to influence behaviour, the latter most probably by stimulating the production of gonadal hormones. However, the experiment is not conclusive on the latter point, because it is possible that the gonadotrophins may have affected behaviour directly, since it has been shown that in *Fundulus* part of the spawning behaviour is induced by the direct action of gonadotrophins (see p. 194).

The data given in this section strongly suggest that the secondary sexual characters and the reproductive activities of the 3-spined Stickleback are also dependent on the activity of the pituitary-gonad mechanism.

3. THE INFLUENCE OF EXTERNAL AND INTRINSIC FACTORS ON BREEDING

a. *External factors and pituitary activity*

In a number of Teleosts seasonal reproduction is correlated with changes in the histology and physiology of the pituitary (Bock, 1928b; Matthews, 1939a; Buchmann, 1940; Kerr, 1948; Fontaine and Olivereau, 1949; Scruggs, 1951). These findings indicate that

external conditions may directly affect the activity of the pituitary, though they do not exclude the possibility that the changes might have been caused by an inherent pituitary rhythm (see 3c). In birds it has been shown that there is a restricted period after breeding in which stimulation with light is ineffective (refractory period); RILEY (1936); RILEY and WITSCHI (1938); SCHILDMACHER (1939); CLARK *et al.* (1937); BURGER (1947); MILLER (1948; 1954); BENOIT, ASSENMACHER and WALTER (1950); MARSHALL (1951); VAUGIEN (1955). The refractory period might be caused by external or internal factors rendering either the pituitary or the gonads (or both) insensitive. On p. 200 literature will be cited in which it is shown that the pituitary most probably is the cause of the refractory period.

In Teleosts no experiments have been made to study the pathway along which the external factors may reach the hypophysis. But in mammals and birds it has been shown, for example, that light is transmitted via the optic nerve (LE GROS CLARK *et al.*, 1938; THOMSON and ZUCKERMANN, 1954; BENOIT and ASSENMACHER, 1953 a,b, 1955). The latter investigators were able to show that in birds light is either transmitted along the optic nerve to the hypothalamus, or penetrates through the tissues of the orbita and thus reaches the hypothalamus. The hypothalamus in its turn affects the pituitary. On the nature of the latter mechanism much controversy exists at the present time and for discussion of the problem the reader is referred to the reviews and studies by BARGMANN, 1954; METUZALS, 1954; BENOIT and ASSENMACHER, 1953a, 1955; HARRIS, 1955.

On the mechanism of temperature influence on the pituitary no data are available. In cold blooded animals it seems likely that the temperature affects the gland directly by influencing the body temperature of the animals. However, it is also possible that the temperature first affects the hypothalamus which in its turn influences the activity of the pituitary.

Very little is known of other factors influencing the activity of the pituitary. But BRENEMANN (1942) has shown that in birds shortage of food caused a decrease in the number of basophilic cells in the pituitary (these cells are the producers of gonadotrophic and thyrotrophic hormones).

b. *External factors and gonadal activity*

Very little data seem to be available on this subject. If there would be a direct influence of external factors on the gonads, then this effect would consist of a process of sensitization, i.e. the external conditions would render the gonads more sensitive to the gonadotrophins secreted by the pituitary.

With regard to the sensitivity of the gonads it has been shown that in a number of animals the gonads are not always equally sensitive to gonadotrophins (Bissonnette, 1938; Riley and Witschi, 1938; Van Oordt and Van Oordt, 1955; Van Oordt, 1956). In all these cases it is possible that the alternation of sensitive and non-sensitive periods has been caused either by an inherent testis-mechanism or by the influence of external conditions. Only in one case has it been shown that an external factor influenced the sensitivity of the gonads. Tolberg (cited by Tuchmann Duplessis in the discussion of the paper of Smith, 1955) demonstrated an increased sensitivity of the gonads of frogs to gonadotrophins by keeping the animals cold. At 4° C ovulation occurred with 4 i.u. of gonadotrophins, but at higher temperatures 12–41 i.u. were required to produce the same effect.

However, other investigators (working with birds) were unable to show a reduced sensitivity of the gonads at a time of the year in which they are generally expected to be less sensitive (Clark *et al.*, 1937; Schildmacher, 1939; Benoit, Mandel, Walter and Assenmacher, 1950). They showed that birds at various times in their refractory period (that is the period after the breeding season) were able to respond to gonadotrophins by increasing their testis-weight. This means that the refractory period most probably is not caused by a process of desensitization of the gonads, but by a reduced activity of the pituitary (see also Greeley and Meyer, 1953).

Though there is as yet little agreement on the subject it seems that in some animals there might be an alternation of sensitive and non-sensitive periods in the gonads. This cycle may either be due to an inherent testis mechanism, or to the influence of external factors. In the latter case the external condition may affect the gonads directly or via stimulation of the hypothalamus. Nowakowski (1950) has shown that the hypothalamus might regulate the sensitivity of the gonads for the luteinizing hormone.

With regard to the pathway along which external conditions may affect the gonads directly nothing seems to be known, but it might be supposed that the former conditions act via the intermediation of the hypothalamus, though in cold blooded animals it is possible that temperature may have a direct effect because it influences the body temperature.

c. *Intrinsic factors affecting breeding*

In a few animals there are indications that they possess an intrinsic rhythm that regulates the timing of the breeding season, without the help of external factors (see p. 154). In other animals it is known that an internal factor, either in co-operation with external factors or not,

terminates the breeding period, though they need certain external stimuli to start breeding again.

The question now arises what kind of intrinsic factors are responsible for the above mentioned internal regulation. It seems very likely that the latter is caused by an inherent property of either the gonads or the pituitary, or perhaps of both acting at the same time. In this respect it is thought that the gland (pituitary or gonad) can only be active during a limited period of time, after which it becomes exhausted and enters a refractory period in which it cannot be stimulated further, either by internal or by external stimuli. In case an intrinsic factor is also responsible for the onset of breeding it is thought that after the refractory period the gland enters a new period of activity all by itself, i.e. without the help of any external factor. COOMBS and MARSHALL, (1956) have shown that in hypophysectomized birds a new generation of connective tissue cells (forming the Leydig cells) arises, which points towards an inherent testis mechanism. The authors, however, were not able to show whether or not the newly formed Leydig cells could also actively secrete in the absence of the pituitary. VAN OORDT and VAN OORDT (1955), and VAN OORDT (1956) have shown that in *Rana temporaria* there is an internal factor which causes cyclical changes in the sensitivity of the germinal epithelium to gonadotrophic hormones.

It is known that other glands (e.g. thyroid and adrenals) also influence the activity of both pituitary and the gonads, but in view of the present scanty knowledge on this subject it seems to be very far fetched to ascribe the above regulation to one of these hormones.

4. GONADAL PHASES AND THE WAY IN WHICH EXTERNAL AND INTRINSIC FACTORS MAY AFFECT THESE PHASES IN THE THREE-SPINED STICKLEBACK

a. *The phases of gonadal development*

In the previous chapters the existence of different phases in gonadal development until maturation have been demonstrated. These phases were 0, 1a, 1b, and 2, the latter phase ending at maturity. It is thought that in under-yearling fish the gonads develop from phase 0 to maturity in the sequence given above, while in adult animals phase 0 is no longer present and gonadal development starts with phase 1a.

A few words must be said on what is meant by the word phase. In the present investigation a phase is a physiological condition that can or cannot be stimulated to develop to a further phase by external factors. It is evident that it is a physiological and not a histological phase, particularly when it is remembered that the different phases

are similar in the two sexes, which means that the same physiological phase comprises different tissues in each sex.

However, it is very tempting to try to identify the physiological phases with known histological phases. This will be done for phases 1b and 2 in the male.

In the male Stickleback it is known that testis development comprises at least two different tissues, namely the germ epithelium and the interstitial tissue (Courrier, 1922; Craig-Bennett, 1931). The latter author showed that spermiogenesis starts soon after the end of the breeding season (thus in July and August) and proceeds until December or January, when the process is completed. However, at the latter time the interstitium has not yet started to develop. Development only begins in January and continues until the middle of April, when the breeding season starts.

Now it is suggested that phase 1b may be identical with the phase in which spermiogenesis takes place or might be completed, but in which the interstitium has not yet developed. Phase 2 might be compared with the phase in which spermiogenesis is completed and in which the interstitium has started to develop. These assumptions are in agreement with the fact that in December, January, and February some of the animals in nature are still in phase 1b, while others have already entered phase 2. The number of animals in phase 2 gradually increases in the course of time (see tables 1 and 2). Future histological work will probably show whether these assumptions have been right or not. At the moment it is not possible to compare other physiological phases with histological phases, because in the 3-spined Stickleback no more histological phases are known.

It is emphasized here that the physiological phases have been called gonadal phases, because they were demonstrated by whether or not gonadal development had taken place under the influence of external conditions. However, it must be remembered that gonadal development depends on the presence in the blood of pituitary gonadotrophins (though this may not hold for all phases, see Coombs and Marshall, 1956). Therefore, the possibility exists that all or some of the phases may correspond with certain phases in the pituitary and not with gonadal phases. Future work should take these considerations into account and both the pituitary and the gonads must be investigated in any attempt to compare the above phases with possible histological phases.

b. *Gonadal phases and their reaction to external stimuli*

Table 5 gives the different physiological phases and the way in which they are defined by their response (either positive or negative) to the

various external conditions to which the animals have been exposed in the present investigation. A positive response means that the gonads developed to the next phase or further.

TABLE 5

Phase	*day length: 16 h. temp.: 20° C*	*day length: 8 h. temp.: 20° C*	*day length: 16 h. temp.: 4°–6° C*	*day length: 8 h. temp.: 4°–6° C*
0	—	+	+	+ (?)
1 < 1a	+	—	—	+
1 < 1b	+	—	+	—
2	+	+	+	+

Table 5. The phases of gonadal development and their reaction to different external conditions.

The table shows that some phases respond to a certain external condition, while others do not. In case the animals respond it is possible that the external condition either affected the activity of the pituitary and thus indirectly that of the gonads, or that it directly influenced the sensitivity of the gonads for gonadotrophins, or that it affected both mechanisms at the same time. In case the animals did not respond, the above reasoning can also be given, to explain the fact that the external condition did not affect the activity of the gland (s). None of the experiments described so far can give information on the question which of the three possibilities is true. But the next experiment was designed to obtain more information on this point.

In Chapter VI it has been shown that animals in phase 1 do not respond to 8 hours day length and 20° C, even when kept under the same condition for over one year. Now if in this case the absence of a response is due to an insufficient secretion of gonadotrophins by the pituitary, it might be expected that administration on extra gonadotrophic hormones would cause the animals to mature. An experiment based on this supposition is described below.

Experiment 40. The animals used were under-yearling fish that had hatched in the laboratory and had been reared in the aquarium-house under almost natural conditions (see fig. 1, 1954). On June 14 they were exposed to 8 hours day length and 20° C. No animals matured before October 26. On that day they were divided into two groups, one receiving a treatment with gonadotrophins and the other serving as a control. The experiment was carried out in the same way as the previous one. There were 3 experimental and 4 control fish. The amount of hormones administered was as follows:

26/10–31/10	2 hours,	312 i.u.	Pregnyl
1/11–14/11	2 hours,	625 i.u.	Pregnyl
15/11–19/11	3 hours,	750 i.u.	Pregnyl
20/11– 5/12	3 hours,	1000 i.u.	Pregnyl
6/12–23/12	4 hours,	1000 i.u.	Pregnyl
24/12–31/12	3 hours,	625 i.u.	Pregnyl
1/1 –11/1	3 hours,	875 i.u.	Pregnyl
12/1 –24/1	5 hours,	1555 i.u.	Pregnyl
25/1 – 1/2	5 hours,	1777 i.u.	Pregnyl plus
		1620 i.u.	Gestyl
2/2 – 5/2	5 hours,	2000 i.u.	Pregnyl plus
		1620 i.u.	Gestyl

The reader will see that until 24/12 the amount of hormone was increased gradually (either by a longer immersion of the fish in the hormone-bath, or by a greater dose, or by both). During this time the animals became notably more silvery, while in one fish the eyes developed a bluish shine (first indications of developing secondary sex characters). However, no further increase in nuptial coloration was observed after 6/12. Because it was feared that the increase in the amount of hormone had been too fast, the amount was then reduced during about one week. On 1/1 it was gradually increased again. But no further signs of gonadal development could be noticed until 25/1 and it even seemed that the silvery shine in the fish disappeared, while the blue in the eyes of one of the animals also faded away.

Since Pregnyl mainly contains the I.C.S.H. component it was now decided to give the animals in addition a preparation containing chiefly the F.S.H. component (Gestyl, made from pregnant mare serum). It was hoped that the joint action of both components would give a response (at the same time the dosage of Pregnyl was increased). But this treatment also failed and when the experiment was broken off on February 4, no maturation had taken place, though some gonadal development may have occurred, since in the beginning the animals showed some traces of nuptial coloration. In Part II more evidence will be given that at least some gonadal development had taken place (see p. 269). However, this development did not go as far as phase 2, for then the animals would have responded to the external condition of 8 hours day length and 20° C (see table 5). Also the control fish did not respond during the experiment, showing that they too were still in phase 1.

Both experimental and control animals were, after the end of the above experiment, left under the same condition until July 20, but none of them matured. However, when at that date they were trans-

ferred to 16 hours day length and 20° C all four control fish matured, on the average 26 days after the transfer (range 22–31). Two of the experimental fish matured, 37 and 40 days respectively after the transfer (the third animal died after about one month). It seems that the hormone-treatment had affected the fish somewhat unfavourably, though the number of animals used is too small to be sure. Probably the hormone-treatment caused the formation of anti-hormones or anti-bodies, which unfavourably influenced the response to 16 hours day length and 20° C (see also below).

The experiment shows that administration of mammalian gonadotrophins to young fish in phase 1 does not induce gonadal development until phase 2 under 8 hours day length and 20° C. In experiment 39 it has been shown that the gonadotrophins used were able to induce maturation in young animals in phase 2. The conclusion, therefore, might be that under the condition of 8 hours day length and 20° the gonads are not sensitive to gonadotrophins and thus cannot develop and mature.

However, there are several reasons to be very careful in regarding the result of this experiment as conclusive. In the first place it is possible that mammalian gonadotrophins are only a very weak stimulus for the induction of maturation. It might be sufficiently strong to accelerate maturation in animals in phase 2 (which already produce a considerable amount of gonadotrophins themselves) but it is possible that it is not strong enough to cause development in animals in phase 1 (which do not yet produce so much gonadotrophic hormones themselves).

It has been shown that in a number of Teleosts administration of mammalian gonadotrophin had little or no effect (Fleischmann and Kann, 1952; Koch and Scheuring, 1936; Ihering and Azevedo, 1937; Creaser and Gorbman, 1939; Johnson and Riddle, 1939; Hasler, Meyer and Field, 1939). A positive effect of mammalian gonadotrophins has been reported by Wunder, 1931; Craig-Bennett, 1931; Owen, 1936; Schreiber, 1936; Ramaswami and Hasler, 1955; and the author (experiment No. 39). Nevertheless there are indications that mammalian gonadotrophins have a weaker effect than the species specific hormones.

In the second place it is possible that the long treatment with gonadotrophins has resulted in making the animals insensitive to these hormones, by the formation of anti-hormones or anti-bodies (Collip and Anderson, 1934; Collip, 1938). The fact that this effect did not show in the first experiment (39) was probably due to the rather short response-time in these animals during which the formation of anti-hormones or anti-bodies could not take place.

Though the above experiment gives some indication that insensitivity of the gonads to gonadotrophins (due to the external condition) might

be the cause of the inhibition of maturation in phase 1 under 8 hours day length and 20° C, the above considerations make it clear that more experiments are needed before a definite conclusion on this subject can be made.

To end this section a few remarks will be made on some other results obtained in the present investigation. The experiments in Chapters IX and X have shown that in the Sticklebacks there is a refractory period in which the animals do not react further to stimulating external factors (non-reproductive period). These experiments showed that in this case the refractory period was caused by the co-operation between an intrinsic factor and the external condition.

On p. 200 an example was given in which it was shown that a period of low temperature increased the sensitivity of the gonads to gonadotrophins (experiment of TOLBERG). In the Stickleback the favourable influence of a period of short day length and low temperature on development of phase 1a (Chapters VI and VII) might also be caused by a sensitizing effect on the gonads.

No investigations have yet been made on the pathway along which the external conditions may affect the pituitary-gonad mechanism in fishes. Therefore, no further suggestions on this subject can be made than those given in section 3.

XII. CONCLUSION

Part I of the investigation will be concluded by a description of the way in which the breeding season of the 3-spined Stickleback is timed in nature. The main evidence for this theory is given by the experiments described in the foregoing chapters. Some evidence came from observations made by other investigators on the same animal or on other species of Vertebrates. In order to indicate where the evidence that is given in the theory comes from, the above sources of information will be marked. Evidence coming from personal experiments will be marked by ◆, that from other investigators on the same animal by ○, and that obtained from studies in other species of Vertebrates by *.

Let us begin at the time when the gonads of adult fish start to redevelop, i.e. in the period following the breeding season.

The breeding season ends in the second part of June or the beginning of July (◆; ○). Gonadal recrudescence, beginning with phase 1a (◆) starts shortly after the end of the reproductive period, namely in July or August (○). Gonadal development in phase 1a can be favourably affected by a short day length combined with a low temperature (i.e. by conditions occurring in autumn) and phase 2 can be reached around December and January (◆).

If, for some reason, phase 1a has developed before the external conditions become favourable for further development (short day length and low temperature) the animals will become exposed to the above condition in phase 1b, which cannot be favourably affected by this condition (◆). The result will be that the development of the gonads remains stationary in phase 1b in autumn and winter (◆). This phenomenon prevents an untimely maturation (in autumn) of Sticklebacks and is responsible for the fact that in January and February animals can be found that are still in phase 1b (◆). Phase 1b is most probably identicial with the histological phase in which spermiogenesis is completed but the interstitium has not yet started to develop (○). However, as soon as the day lengths become long, i.e. in the first months of the year, the gonads are able to develop from phase 1b to phase 2 and further to maturity (◆).

In March all animals have reached phase 2 (◆) and further development is favourably affected by long day lengths (◆, ○) as well as high temperatures (◆, ○). The gradual increase as such of day length and temperature has no influence on the rate of gonadal development (◆). The latter depends on the summation of the day lengths and probably of the temperature, rather than on the gradual increase (*). There is no intrinsic factor which, in the absence of certain external factors, causes the onset of the breeding (◆).

Breeding of the Sticklebacks begins (in Holland) around the middle of April (◆, ○).

During the reproductive season the animals are exposed to long day lengths and high temperatures and this external condition, together with an intrinsic factor allows breeding during a period of about 3 months (◆). This means that the reproductive season lasts until the middle of July (◆; ○).

So much for the timing of breeding in adult Sticklebacks. We must now discuss the regulation of breeding in young animals. The first young Sticklebacks hatch at the end of April and the last sometime in July. All young animals, and particularly those that have hatched early in the season, live under favourable circumstances, namely a high temperature and abundance of food. This might cause a rapid growth and gonadal development were it not that in these animals (phase 0) the long day length and the high temperature inhibit (at least partially) growth as well as gonadal development (◆). It has been shown that under artifical conditions young fish can be induced to mature within 5 months of hatching (◆). The above inhibition of growth and gonadal development in summer, therefore, prevents maturation of young fish in autumn, which of course would be unfavourable for the survival of the species. However, as soon as the day

lengths become short and the temperatures low (i.e. in autumn) the inhibition of growth and gonadal development is abolished (◆) and the animals develop to phase 1a (◆). The rest of the cycle proceeds in the same way as that decribed for the adults.

Though the results obtained in the present investigation allow the most detailed picture of the mechanism timing seasonal breeding that has been given until now, it nevertheless still shows many gaps that can only be filled by more detailed experimental work. The different points that need further investigation cannot be discussed here, since they have already been mentioned in various foregoing chapters.

Summarizing the main results obtained in the present investigation it can be said that there is no doubt that in the 3-spined Stickleback the timing of the breeding season is controlled by a delicate interaction between various external and internal factors. External factors which have been shown to play a rôle are day length and temperature, which in some cases act independently of each other, while in other instances only particular combinations of these two external factors can stimulate (or inhibit) development. The intrinsic factors playing a rôle in the timing of breeding cannot be defined, but it is very likely that they are properties of the pituitary or of the gonads. Other intrinsic factors may (also) be involved but at the moment nothing seems to be known on this subject.

XIII. SUMMARY

Chapter I. The present study deals with problems concerning the causation of seasonal breeding cycles. In the 3-spined Stickleback the influence on gonadal development of various external and internal conditions at different times in the annual reproductive cycle has been studied experimentally. Attention has been paid not only to factors causing the onset of breeding, but also to those terminating the breeding season.

Chapter II. Information is given on the material, method and technique employed. As criterion for the attainment of maturity nestbuilding behaviour was used in the males and oviposition in the females.

In order to facilitate the understanding of the results of the experiments, a preliminary description is given of the various phases of gonadal development. Experiments justifying the distinction of these phases are discussed in various chapters.

The phases are 0 (only in under-yearling fish); 1a; 1b and 2.

Gonadal development proceeds in the sequence given here and maturity is reached at the end of phase 2.

Chapter III. The influence of temperature on certain phases of gonadal development and sexual behaviour is described. The experiments demonstrate that at long day lengths the rate of gonadal development in phases 1b and 2 increases with temperature. At short day lengths only the rate of gonadal development in phase 2 increases with the temperature, phase 1b not being affected. No essential difference between the responses of males and females has been observed. In nature the above phases occur between December and May.

At long day lengths gonadal development (in phases 1b and 2) can even take place at the very low temperature of 4° C. It is argued that this may be of importance for the timing of breeding in nature where very low temperatures sometimes continue until the middle of March, whereas the days have become quite long by that time. The lowest temperature at which males will build nests is about 5° to 6° C.

Chapter IV. The influence is described of day length and light intensity on certain phases of gonadal development. The rate of gonadal development in phases 1b and 2 increases with day length, both at high and at low temperatures.

Light intensities above 25–30 f.c. do not accelerate gonadal development any more than an intensity of 25–30 f.c. However, lower intensities retard the maturation process.

Chapter V. The experiments deal with the widely-held opinion that the gradual increase as such of day length and of temperature would be of primary importance for the attainment of maturity in animals breeding in spring. A comparison between the influence on gonadal development between December and May (phases 1b and 2) of gradual increases in day length and temperature (separately as well as in combination) and that of a sudden increase shows that of the two the sudden increase has a much stronger accelerating effect on maturation.

Chapter VI. The existence is demonstrated of critical external factors in the absence of which Sticklebacks are not able to attain maturity. Short day lengths combined with high temperatures prevent animals in phases 1a and 1b from attaining maturity, even when they are kept under this condition for more than one year. However, when these fish are subjected to long day lengths and high temperatures they mature within a short time. Therefore, the latter is a critical condition for maturation.

A short day length combined with a low temperature followed by a short day length and a high temperature abolishes the above inhibition of maturation in fishes being in phase 1a. This only holds for animals in phase 1a; in phase 1b the inhibition is continued. Therefore, a short day length combined with a low temperature is, under given circumstances, also a critical condition for maturation.

In the 3-spined Stickleback maturity can only be attained under the influence of certain external factors. In the absence of these factors no intrinsic factor or rhythm is able to induce maturity.

However, some internal factors have a modifying effect on the initiation of maturation. In animals that have never spawned before, the latency of the response to the favourable external condition is longer than in those that have spawned at least once. Further it has been demonstrated that under the above mentioned condition of short day lengths combined with high temperatures the degree of responsiveness to the favourable external condition fluctuates cyclically.

The possible existence of other critical environmental factors is considered.

The function in nature of the two critical external conditions for maturation described above is discussed. It is concluded that these conditions secure breeding in spring in spite of possible variations in climatological conditions.

Chapter VII. Experimental evidence is given that the influence of a given external factor(s) depends, 1. on the internal state of the fish and 2. on the other external factor(s) present.

The former is proved by the fact that the influence of external conditions on gonadal development varies with the time elapsed since the termination of the previous reproductive season. In other words, the response of the gonads to certain external conditions depends on the phase of gonadal development at the beginning of the experiment. It is shown that the timing of the onset of the natural breeding cycle by external conditions is not effected only in the period between midwinter and spring, but that this timing starts immediately after the end of the previous breeding period. This implies that external conditions during autumn also play a rôle in the timing of reproduction, even though we are dealing with a species breeding in spring.

The second influence is illustrated by the fact that at one day length (or temperature) gonadal development may be stimulated or inhibited, depending on the temperature (or day length respectively) with which it is combined.

Chapter VIII. It is shown that maturity can be induced in young animals within 4 to 5 months after hatching by exposing them to a

long day length combined with a high temperature. In this case the nuptial coloration of the males lags far behind gonadal development.

However, there is one phase in gonadal development (phase 0) in which young fish do not respond to the above external condition, not even when they are kept under this condition for over one year. In phase 0 the animals are usually shorter than 2.2 cm. This phase is not only characterized by the absence of a gonadal response to the external stimulus (most probably due to insufficient production of gonadotrophins), but also by a slow rate of growth (most probably caused by an insufficient secretion of growth hormone). Perhaps, the pituitary of animals in phase 0 is not able to respond to the external stimulus in the way it does in animals in phases 1a, 1b or 2. Having once spawned, the fish never come into phase 0 again.

It is argued that the absence of gonadal response and the slow growth rate under long day lengths and high temperatures may play an important rôle in the timing of breeding in nature, for they prevent a precocious maturation in autumn of animals hatched in spring and summer.

The inhibiting influence on gonadal development of long day lengths combined with high temperatures can be abolished by temporary exposure of the young fish to either a long day length combined with a low temperature, or to a short day length combined with a high temperature. A short day length and a low temperature both occur in autumn and in this way the onset of breeding in young fish in spring is secured.

Chapter IX. Experiments are described in which the duration of the breeding period under various external conditions was determined.

The average duration of the reproductive period at the long day length and high temperature used was 91.4 days in the males and 60 days in the females. The latter can spawn as often as 15 times per reproductive period.

The duration of the breeding period at the short day length and high temperature used was on the average 45.8 days in the males and 6.6 days in the females.

The fact that breeding always comes to an end even under constant external conditions suitable for the occurrence of reproduction, proves that an intrinsic factor is involved in terminating the breeding season. The fact that the length of the breeding period is influenced by external factors shows that the above intrinsic factor interacts with environmental stimuli.

The duration of the reproductive period in animals that had first been exposed to a long day length together with a high temperature

and subsequently to a short day length combined with a high temperature was on the average 57 days in the males and 11.1 day in the females. This indicates that in the females (and probably also in the males) the previous exposure to a long day length combined with a high temperature had a favourable after-effect on the duration of breeding. Again it can be concluded that the termination of the reproductive season is ended by the interaction between intrinsic and external factors.

In nature, where the animals are exposed to long day lengths and high temperatures from the beginning of reproduction in the middle of April, breeding in males also lasts about three months, namely until the middle of July.

There was no difference in the duration of the breeding periods of males that had spawned at least once and of those that had never spawned before.

Chapter X. The experiments in which fish were kept under constant conditions of a long day length and a high temperature both males and females showed an alternation of reproductive and non-reproductive periods. This can be explained by assuming that after a refractory period (caused by intrinsic and external factors in co-operation) the animals become sensitive again to the external condition which induces gonadal recrudescence and maturity.

At a short day length combined with a high temperature no such alternation of reproductive and non-reproductive periods occur, because the early phases of gonadal development (1a and 1b) cannot develop to phase 2 and from there to maturity under this external condition.

Chapter XI. The rôle of the pituitary-gonad system in the physiological mechanism responsible for the timing of the reproductive season is discussed. In gonadectomized males nestbuilding did not occur anymore and the secondary sex characters disappeared or remained undeveloped.

An experiment is described in which a mammalian gonadotrophic hormone induced an increase in aggressive and sexual activities and also a precocious onset of maturity.

The way in which external and internal factors may affect the pituitary-gonad system is discussed.

Animals prevented from maturing for a long period by keeping them at a short day length combined with a high temperature were treated with mammalian gonadotrophins. None of the treated fish or of the controls matured. This suggests that the gonads are insensitive to gonadotrophins under this external condition. However, it must be

kept in mind that the absence of a response may also be due either to the fact that mammalian gonadotrophins are usually only a rather weak stimulus in fishes, or to the formation of anti-hormones or anti-bodies.

Chapter XII. Mainly on the basis of the results of the experiments made in the present investigation a picture is developed of the way in which the breeding cycle is caused in the 3-spined Stickleback. It shows clearly that the timing of the reproductive season is achieved by a very delicate interaction between intrinsic and external factors.

PART II

THE TIMING OF MIGRATION

I. INTRODUCTION

The problem of migration (to be defined here as the periodic displacement of animals between two areas) generally comprises three aspects: *a.* the causation of migration, *b.* orientation during migration, and *c.* the termination of migration. Each aspect represents a field of study in itself. The first two have been a subject of investigation for many years, but no work seems to have been done yet on the third. The present investigation deals with the causation of migration in the 3-spined Stickleback, a diadromous species (terminology of MEYERS, 1949).

The following survey will deal mainly with migration in fishes, though some remarks will be made on bird migration.

The work of MEEK (1916) and SCHEURING (1929, 1930) contains a wealth of information on a great number of migratory species of fish, particularly on the time of the year in which migration takes place and on the areas between which the animals migrate. The information is based on observations in nature, but the problem of the causation of migration has to be studied experimentally in the laboratory. One of the greatest difficulties in this experimental study is the finding of appropriate criteria which indicate whether or not migration has been induced. Several criteria have been used until now by different authors and the most important will be described here. The first three have been used in the study on fish migration and the last on migration in birds.

1. *Behaviour (rheotropism; general activity).* While migrating many fishes move either with or against the current (negative and positive rheotaxis respectively). In a number of cases the rheotactic responses of fishes under different external conditions have been studied and also the effect of certain hormones on rheotropism have been a subject of investigation. Migrating fish are generally more active than sedentary animals and therefore the activity and the behaviour of fishes have been studied under various experimental conditions.

2. *Osmotic regulation.* A great number of fishes migrate between the sea and fresh water (diadromous) and it could be expected that changes in the osmotic regulation of the animals at the time of migration are involved. To study the rôle of osmotic regulation in migration the concentration of the body fluids is determined in the course of the year, or in relation to changes in the degree of salinity of the external medium. Since it is known that in many cases migration is associated with a hyperactivity of the thyroid gland, extensive studies of this gland and its influence on osmotic regulation have been made.

3. *Salinity tolerance and preference.* These criteria may be closely connected with the previous one, for salinity tolerance and probably also salinity preference may depend on osmotic regulation. However, by using the present criteria not the osmotic regulation itself, but the behaviour of the fish when exposed to different salinities, is studied. In the case of tolerance the survival time of the animals is determined, while in the case of preference the fish are allowed to seek the medium they like best. In the former case the fish play a passive rôle, while they have an active rôle in the latter. In the present study the criterion of salinity preference was used and its method and technique will be described in the following chapter.

4. *Marking and recovering; deposition of fat reserves; restlessness.* These criteria are only used in birds. By using the first criterion the animals are first subjected to various experimental conditions and subsequently released. From recoveries of marked birds conclusions can be drawn on whether the animals had moved away from the place where they were released and if so in what direction (indicating spring and autumn migration) they had moved.

By subjecting the animals to experimental conditions it was observed that in some cases the birds became much heavier by depositing fat reserves. This also occurs in nature prior to migration. Therefore, the deposition of fat is very often used as a criterion for the induction of migration.

Migratory birds kept in cages become restless at the time of migration and orientate their movements in a direction corresponding to the direction of migration. This criterion has also often been used to study migration in birds.

On the basis of observations and experimental work several hypotheses explaining the causation of migration have been postulated. The most important views on this subject will be briefly mentioned below, while for details the reader is referred to the discussions at the end of some chapters of the present paper.

In a great number of cases migration is correlated with either

development or regression of the gonads and since the pioneering work of ROWAN (see his review, 1938) on bird migration many investigators believe that migration is *caused* by the gonadal cycle. Objections have been raised against this theory, notably by PUTZIG (1937, 1938, and 1939); WOLFSON (1945, 1952); FARNER (1950); FONTAINE and KOCH (1950); FONTAINE (review, 1954). These investigators hold that other physiological changes than those of the gonadal cycle are responsible for the causation of migration.

WOLFSON believes that increasing day lengths in the early months of the year induce a change in the physiological condition of the birds (characterized by deposition of fat reserves) which causes migration.

FONTAINE and KOCH believe that the internal physiological change is not so much due to external conditions, but rather to an intrinsic property of the animals. External factors would only play a secondary rôle in inducing the actual moving of animals in the right physiological condition. In fishes the intrinsic change would be a modification of the neuro-endocrine system (particularly of the hypophyso-thyroid mechanism) which upsets the equilibrium between the organism and its environment, or renders the animal more sensitive to external changes.

HOAR (1951b, 1953) believes that externally controlled changes in the endocrine system (including the hypophyso-gonad system) cause changes in the behaviour of the fishes which are responsible for the initiation of migration.

The aim of the present investigation was to study the rôle of external factors, gonadal and thyroid activity in the timing of migration. In Part I it was shown that external factors are responsible for the onset of breeding and partly responsible for the termination of the reproductive season. Animals used in these experiments were also tested for their salinity preference (criterion for migration). In this way information was obtained on the relation between external factors, gonadal cycle and migration. Later the influence of external conditions was also studied in gonadectomized animal in order to determine the possible rôle of the gonads in the causation of migration. Some experiments were made to study the effect of gonadotrophic hormones on salinity preference. Finally the effect of thyroxine and thiourea (thyroid inhibitor) on salinity preference was determined in normal as well as gonadectomized animals, in order to obtain information on the rôle of the thyroid gland in migration.

II. MATERIAL, METHOD AND TECHNIQUE

1. MATERIAL

The 3-spined Stickleback that was used in this study lives in the sea during winter and migrates in the early months of the year to fresh water to breed. At the end of the breeding season it migrates back to the sea. The exact times of the onset of spring and autumn migration are not known, but a rough estimation can be made on the ground of available observations.

LEINER (1930, 1931) states that in northern Germany spring migration takes place during April and May. IKEDA (1933) found that in Japan spring migration occurs between the middle of February and the end of March, while VAN IERSEL (1953) mentions that in Holland migration begins at the end of March and the beginning of April. CRAIG-BENNETT (1931) observed migration of the Stickleback in England in the second half of April and the first part of May. In all cases migration took place about one or two months prior to the onset of breeding.

However, information given to the present author by fishermen shows that in Holland the onset of migration takes place much earlier than mentioned above. The first animals have been caught while entering sluices in the sea-dykes as early as January. Data from the fishmarket at Zoutkamp (northern Holland) show that in 1943 the catching of Sticklebacks (used for the production of fish meal and as food for ducks) became of commercial importance on February 11. This means that smaller numbers must have entered fresh water even before this date. The biggest captures at this place were made between February 20 and March 10, while the fishing lasted until the middle of April. Near Den Helder (northwestern Holland) animals entering fresh water have been caught in the last week of January, while the biggest captures are reported in February and March.

Generally the number of fish entering the sluices does not seem to be correlated with a particular type of wheater, for large schools of migrating fish have been observed under all kinds of wheather conditions. However, it has been noticed that during a period in which there was a sudden increase in temperature, the number of migrating fish caught in the nets was considerably larger than during the preceding cold spell.

The conclusion that can be drawn from the above data and observations is that in northern Holland migration may start as early as January and may last until May, with a maximum activity in February and March.

With respect to the seaward migration in autumn less data are

available. While spring migration may be carried out by thousands of fish entering sluices at the same time, the autumn migration is far less conspicuous. Many fishermen even believe that only young fish that hatched in spring migrate to the sea and that all adults die after spawning. However, the fact that in autumn adult fish can be caught again in the sea refutes this idea.

Leiner (1930, 1931) states that the breeding season lasts until July, while seaward migration would start in September (data for northern Germany). However, his last observation is based on the dates of arrival in the sea, which means that the onset of migration must have taken place earlier, since the fish had to cover some distance before reaching the sea. Craig-Bennett (1931) states that in England breeding lasts until July, while he observed that at that time animals were already migrating back to deeper water. In Holland no observations on the beginning of seaward migration are available, but fishermen have reported that the first animals to be caught in the sea appear around October and are present in abundant quantities during November and December. This implies that the onset of migration must have taken place in some animals earlier than October.

In 1955 the return to the sea took place much later than in the preceding year, most probably because of the very mild weather during September and October.

It can be concluded that in Holland seaward migration may start shortly after the end of the breeding season and last until November.

The young fish that hatch in spring may also migrate to the sea, but there are indications that at least some of them winter in fresh water. Craig-Bennett (1931) believed that under-yearling fish did not undergo seaward migration. However, among the animals caught in spring, while entering sluices on their way to fresh water, there were many whose length indicated that they must have been under-yearling fish (personal data, unpublished). On the other hand in 1955 young animals were caught in fresh water around Groningen in November, December and January. The mean length of these fish was 3·5–3·9 cm, which indicates that they must have been under-yearling fish. Though the ditches in which they were caught had open connection with canals leading to the sea, these fish had evidently not carried out the seaward migration.

Sticklebacks living in fresh water connected to the sea do not always winter in the sea, but may stay in brackish inland waters close to the sea. Animals living in fresh water that has no connection with the sea are reported to migrate to deep water in autumn and to return to shallow water in spring (Craig-Bennett, 1931; Meek, 1916; Scheuring, 1929, 1930).

The Sticklebacks used in the experiments on migration were caught in the north of Holland and belonged to three different races distinguished by BERTIN (1925), namely *Gasterosteus aculeatus forma trachura*, *forma gymnura*, and *forma semi-armata* (for a description of the races see p. 112). For details on the distribution and physiological properties of the races see BERTIN (1925) and HEUTS (1947 a, b, 1949).

2. METHOD

First a short introduction must be given on the concept underlying the method used here for the study of migration. GROEBBELS (1928) distinguished two stages in the onset of migration, the "Zugdisposition" and the "Zugstimmung". He thought that different primary factors would bring the animals into a certain physiological condition ("Zugdisposition") after which factors of secondary importance would activate the "Zugstimmung", under the influence of which the animals actually migrate (see also FARNER, 1950). In terms of our present knowledge this would mean that first an internal change has to take place, inducing migration-motivation (caused by external and/or intrinsic factors), after which appropriate external factors are able to activate the migration-drive, which causes the migration movements. The word "drive" is used in the sense of "the total quantity of information supplying a behaviour" (BAERENDS, BROUWER and WATERBOLK, 1955). The above distinction will be used in the present investigation and is in agreement with the modern concept on the causation of behaviour.

I do not agree with VERWEY (1949) who thinks that the above distinction should be discarded. According to him two groups of external factors (periodic and non-periodic) influence the time of the onset of migration.

In the study on Stickleback migration a change in salinity preference of the animals was used as criterion for the induction of internal migration-motivation. The choice of this criterion will be justified in Chapter IV.

In case the animals start to prefer fresh water it is supposed that they have come into a physiological condition which, under the influence of appropriate external conditions, would induce actual migration from the sea to fresh water.

In case the animals start to prefer salt water, it is supposed that they have come into a physiological condition which, under the influence of appropriate external conditions, would induce actual migration from fresh water to the sea.

In this concept it is assumed that under certain experimental

circumstances the Sticklebacks can be internally motivated for migration (migration-disposition), though the migration-drive may not be activated because the external conditions are not appropriate. In birds this assumption can be illustrated by the following example. It is known that bad weather conditions can inhibit migration. In this case the migration-drive is not activated, though the animals are no doubt still in migration-disposition, since they will start to move again as soon as the weather improves.

The experiments were carried out in such a way that the animals first underwent a certain treatment (exposure to different external conditions; gonadectomy; administration of hormones, etc.) after which their salinity preference was tested.

3. TECHNIQUE

a. *Description of the experimental set-up*

The tests were carried out in wooden troughs that were 100 cm long, 15 cm wide, and 13 cm deep (inner dimensions). They were made watertight and covered with a special waterproof varnish. The colour of the inside of the troughs was a dark brown with patterns of different shades corresponding to the grains of the wood. These patterns were not uniform and varied within each trough and between the different troughs used.

The troughs were divided into four watertight compartments (each 25 cm long) by glass plates fitted into the bottom and walls (see fig. 9).

Fig. 9. Trough used for salinity tests. Compartments on the left: fresh water; compartments on the right: salt water. The density of the dots indicates the concentration of salt water. For further explanation see text.

The plates were 10 cm high and thus a little lower than the troughs. Two adjoining compartments at one end were filled with fresh water and the other two with sea water. The salinity of the outermost of the two salt water compartments was always made higher than that of the innermost in order to obtain a gradual salinity gradient in these two compartments. The degrees of salinity used will be mentioned later. To make a connection between the four compartments a layer of fresh water was allowed to flow over the top of the glass plates. This was achieved by letting the water flow very gently into the outermost

of the two fresh water compartments, from where it spread gradually over the four compartments (see fig. 9). This layer of fresh water was made as thin as possible (not more than 2 cm) but always allowed the fish to pass freely over the top of the plates. The layer of fresh water caused a mixing of the fresh and salt water, but in section b it will be shown that the degree of salinity of the salt water compartments always remained higher than that of the fresh water compartments.

The water used for the experiments was stored in big battery jars about 3–16 hours before the beginning of the tests. During this time it was well aerated and reached the required temperature. Great care was taken that the temperature of both the fresh and salt water in the troughs was the same (differences less than 0.05° C), in order to be sure that the preference of the animals could not be attributed to temperature instead of to salinity differences. If possible the fish were tested in water of about the same temperature as that of the aquaria in which they were kept during the experimental pre-treatment. Sometimes, however, this was impossible, but it will be shown in Chapter III that this difference in temperature did not affect the salinity preference.

In one test three troughs were used. They were placed parallel on a table that stood in the middle of a room. The table was surrounded by curtains which prevented disturbance from outside. No trouble was taken to make the space as uniform as possible and the animals always had opportunity to orientate themselves to marks both within and outside the troughs. However, the experimental set-up of the salinity tests was such that preference by means of place-orientation was eliminated (see below). The space beween the curtains was brightly illuminated with artificial light (about 40 f.c.), while no natural light penetrated into the room.

During the first year (1952–1953) the tests were made in a room illuminated by natural day light. This circumstance, however, does not seem to have influenced the results.

The observer sat at a high desk, placed at one end of the table outside the curtains and could overlook the troughs through a vertical slit.

The middlemost of the three troughs (A) contained fresh water in all four compartments, while the other two (B and C) contained fresh and salt water. In trough B the sea water was placed in compartments 1 and 2, whereas in C the sea water was located in compartments 3 and 4 (see fig. 10).

Each test consisted of five "sub-tests", in three of which the fish could choose between fresh and salt water. The sequence of the sub-tests was as follows: first the fish (usually 5, but sometimes 2, 3,

or 4) were placed in A for 15 minutes or more and subsequently for the same time in B, in C, back again in B, and finally in A.

There were two reasons for testing the fish in the trough containing only fresh water. In the first place the animals could get used to swimming in the tank and in the second place the results obtained in the fresh water trough could be used for calculation of the frequency-distribution of the fish swimming in the trough (see section d).

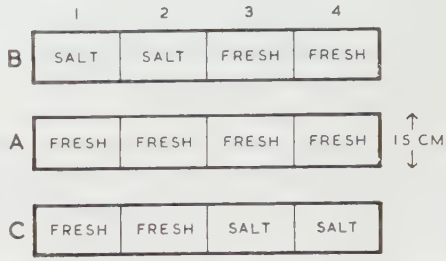

Fig. 10. Location of salt and fresh water in the three troughs used in the salinity tests.

The distribution of the animals over the four compartemnts was noted each 15 sec. during a period of 15 minutes or longer. Since it was possible to make such a note within 7 sec. it was decided to test two groups of fish at the same time. The sequence of the two series ("double test") was as follows: 1°, group I in A; 2°, group I in B, group II in A; 3°, group I in C, group II in B; 5°, group I in B, group II in C; 5°, group I in A, group II in B; 6°, group II in A. The water was renewed at the end of each double test.

To transfer the fish from one trough to another they were caught in a very small net that could just contain an adult Stickleback. The net had meshes of about 3 mm and by handling it very carefully the fish could be easily caught without much disturbance or mixing of the water.

At the transfer from one trough to another the fishes were placed in the different compartments according to the following scheme: in A and B two (or three) fish were placed in compartment 2, and three (respectively two) fish in compartment 3. In the following sub-tests the animals were placed in a compartment corresponding in place to the one in which they were captured. In this way the fish that were caught in salt water, for example, were placed in fresh water and vice versa. This was done because the fish would sometimes show a position preference in the troughs and thus did not come into frequent contact with water of different salinity. This might have prevented them from making a real salinity choice and to overcome this difficulty the above procedure was followed. Whenever possible the fish were tested on three successive days.

b. *Salinity*

The sea water used was provided by the Zoological Station at Den Helder. The salinity of the sea water varied from about 31–34.5 pro

mille. In the majority of tests the concentrations of the salt water used were 3/8 sea water in the outermost and 2/8 in the innermost of the two salt water compartments. These concentrations were identical with sea water of about 11.6–13.4 pro mille and 7.8–8.6 pro mille respectively. In the beginning experiments were made with various concentrations of sea water but the results revealed that there was no difference between the results of the tests with a high and a low concentration.

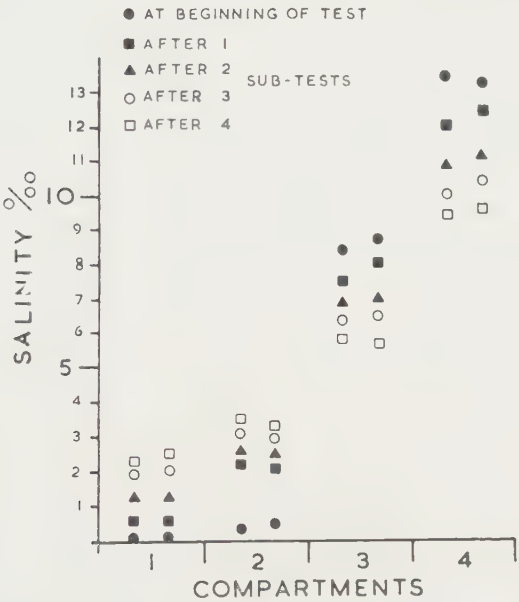

Fig. 11. Salinity in the four compartments of trough B in the course of a "double test" (see p. 222). The points indicate the average salinity obtained in two series of 25 double-tests each.

The above mentioned concentrations of 3/8 and 2/8 sea water was chosen for several reasons. In the first place, as will be further discussed later, different races of Sticklebacks were used and it was known that the races preferred a somewhat different medium with regard to salinity. Therefore, the concentrations were rather low in order not to make the medium repellent to the race which preferred the lowest degree of salinity. In the second place it was the lowest concentration that could be used with good success and the most economical in using the supply of sea water.

During the time that the fish were tested the water in the troughs was mixed by the swimming fish in such a way that the salinity in the

sea water compartments decreased, while that in the fresh water compartments increased. However, the salinity of the former compartments always remained higher than that of the latter throughout the double tests. This is shown in fig. 11.

The salinity was determined in trough B (the one that was used most often, namely 4 times in a double test, see p. 222) by taking samples in the middle of each of the four compartments about 7 cm from the bottom. This was done before the test was started and it was repeated each time a group of 5 animals had been tested in the trough. The points in fig. 11 give the mean degrees of salinity in each of two series of 25 tests. The chlorinity was titrated according to the method of Mohr, while the salinity was calculated from the chlorinity figure by using the formula of Knudsen (1902): Salinity $^0/_{00} = 0.030 + 1.8050$ chlorinity $^0/_{00}$.

c. *Behaviour of the fish in the troughs*

When the fish were placed for the first time in the trough they were able to find their way in this maze within 3 to 5 minutes. In case they had been tested one or more times before, they usually started to swim about immediately after transfer to the trough. In all sub-tests the fish were left for about 3 to 5 minutes in the trough before the records were started.

It was surprising to observe that the Sticklebacks, which usually do not swim very regularly in aquaria, immediately after the transfer to the troughs started to swim quickly from one end to the other. It seemed as if the long narrow troughs stimulated them to swim continuously. While swimming the fish usually proceeded along the side of the trough, though they also used the middle part. The animals could often be seen swimming closely below the surface of the water, frequently pushing their snouts above the surface. In corners of the trough this behaviour often became more pronounced and resulted in the performance of little jumps. Sometimes the jumps were so vigorous that the fish leaped out of the trough. However, jumping only occurred in fishes that were very active swimmers.

Instead of following a straight horizontal line when proceeding from one end to the other, the fish also often swam up and down along the walls, gradually displacing themselves toward the other end. Fishes remaining in one compartment for some time also swam up and down the walls. It is clear that the swimming from one end to the other as well as the swimming up and down along the walls brought the fish into frequent contact with water of different salinity. It is suggested that this kind of behaviour in nature might be very useful, since it would help the animals when entering sluices or passing other

obstacles and perhaps also by bringing them in contact with water of different salinity.

In some cases the animals showed aggressive behaviour while being tested. This behaviour consisted mostly of biting and chasing and when it was performed frequently it seriously hampered the fish in showing a salinity preference. In this case the tests had to be discarded. Since aggressive behaviour is shown particularly often by mature males and females these animals could only rarely be used.

d. *Statistical method*

As has been mentioned before the data on salinity preference were obtained by making records each 15 sec. of the number of fish that were present in the different compartments. This was done in each sub-test during a period of 15 minutes or longer. By using five fishes per group during 15 min., a total number of $5 \times 4 \times 15 = 300$ positions were obtained. These positions were distributed in a certain way over the four compartments. The number of positions in each of the two halves of the trough were added and gave data that could be analysed statistically.

In testing the data the χ^2 test was used. This was chosen because it could be shown that the frequency-distribution of the fish in a trough containing only fresh water was normal, i.e. the number of positions in each of the two halves of the trough was about equal. The figures obtained from the tests made in trough A (only fresh water) showed that in a total of 809 tests (each consisting of two sub-tests) the number of positions in the compartments 1 plus 2 was 235.514 and in 3 plus 4 was 236.302. This makes a difference of 788 positions on a total of 417.816. The value of P of the χ^2 test in this case is larger than 0.35. This means that it can be safely assumed that in a trough containing only fresh water the animals will be distributed equally over the two halves of the trough.

By using the χ^2 test a preference for either fresh or salt water was indicated by P values equal to or smaller than 0.01. In case the value for P was between 0.01 and 0.05 it was assumed that there was a preference, though not very pronounced. P values above 0.05 for either salt or fresh water were assumed to indicate no preference.

e. *Explanation of the figures*

In the various figures the P values of all sub-tests are indicated by marks. Three marks above each other represent the P values of the three sub-tests of one test. In some cases only two sub-tests could be made. The P value in one test are ranked from the top downwards from a preference for salt water to a preference for fresh water. Thus,

the sequence does not represent the sequence in which the sub-tests were made. Another mark above each series of tests represents the P value of the total number of positions obtained in the combined tests of the series (Pt). The meaning of the marks is given in fig. 12.

f. *Gonadectomy*

Gonadectomy in Sticklebacks has been carried out by Bock (1928a), Craig-Bennett (1931), and Ikeda (1933). Gonadectomy in the present investigation was carried out under aethylurethan anaesthesia. A solution of 1 % was used for a few minutes preceding the operation, while during the operation the concentration was 0.5 %. A longitudinal incision of about 5–7 mm was made on the ventral side of the abdomen, a few mm lateral from the medium line between anus and ventral pelvic plate. The testes were taken out (with a pair of fine forceps) by gripping the vasa deferentia as far caudally as possible. Ovariotomy was more difficult, especially when the ovaries were voluminous. In males as well as females one incision was sufficient to remove both gonads. The incision was closed with two stitches of silk thread. Usually 1/4–1/3 of the animals died during the first few days after the operation, but those that survived could be kept in a healthy condition for long periods of time (in one experiment for more than 8 months). The wound closed quickly and the silk threads were either absorbed or thrown off.

III. INTRODUCTORY EXPERIMENTS

In this chapter a number of experiments will be described that were made in order to determine the influence of some (unwanted) factors that were present in almost every experiment and which might have affected the salinity preference.

1. THE EFFECT ON SALINITY PREFERENCE OF DIFFERENCES IN TEMPERATURE BETWEEN THE WATER OF THE TEST TROUGHS AND THAT OF THE EXPERIMENTAL PRE-TREATMENT

In Chapter II it was mentioned that great care was taken to make the temperature of the water in the four compartments of the troughs as equal as possible, in order to avoid interference of a temperature preference in the salinity tests. It was also said that, though in many cases the temperature in the troughs was similar to that of the aquaria in which the animals were kept during the experimental pre-treatment, the temperature in the troughs was often either higher or lower.

Particularly when the temperature in the aquaria was lower than 12° C it was not always possible to test the fish at the same temperature. However, in none of the cases was the difference greater than 5° C.

Since the decrease or increase in temperature to which the animals were exposed when transferred from the aquaria to the troughs might have influenced the salinity preference, some experiments were made on this subject. The acclimatisation of the fish to the temperature of the troughs never lasted longer than half an hour, while the maximal duration of one test did not exceed 3 hours. Thus, the animals were never exposed to the higher or lower temperature for longer than 5 hours daily.

Experiment 1. A group of five Sticklebacks was kept in an aquarium with a temperature of 10° C. They were tested on three successive days at a temperature of 10, 12 and 13.5° C respectively. The results are given in table 6. It can be seen that the fresh water preference is not influenced by the fact that the fish had been exposed to a rise in temperature of 2 and 3.5° C respectively.

TABLE 6

No of experiment	*Temp. in °C during experimental treatment*	*Temp. in °C of tests*	*Difference in temp.*	*Level of significance of salinity preference*								
				P for fresh water			*P no preference*		*P for salt water*		
1	10	10	0	⩽0.01	⩽0.01	⩽0.01					
	10	13.5	+3.5	⩽0.01	⩽0.01	⩽0.01					
	10	12	+2	⩽0.01	⩽0.01	⩽0.01					
2	15.5	15.5	0	⩽0.01	⩽0.01	⩽0.01					
	15.5	10.5	—5	⩽0.01	⩽0.01	⩽0.01					
	15.5	11.5	—4	⩽0.01	⩽0.01	0.05–0.01					
	15.5	12.5	—3	⩽0.01	⩽0.01	⩽0.01					
3	18	18	0				0,101		⩽0.01	⩽0.01	
	18	16	—2						⩽0.01	⩽0.01	0.05–0.01
4	16	16	0						⩽0.01	⩽0.01	0.05–0.01
	16	17	+1						⩽0.01	⩽0.01	0.05–0.01
	16	18	+2						⩽0.01	⩽0.01	⩽0.01

Table 6. The influence on salinity preference of differences in temperature between experimental treatment and salinity tests. The horizontal groups of three P-values represent the preferences in the three sub-tests of each test.

Experiment 2. The fish of this group were kept in an aquarium with a temperature of 15.5° C. They were subjected to a decrease in temperature of 3, 4 and 5° C respectively during three successive days. Table 6 shows that a decrease in temperature also does not influence the preference for fresh water.

Experiment 3. In this case the temperature of the aquarium was 18° C and the fish showed a salt water preference. A decrease in temperature of 2° did not affect this preference (see table 6).

Experiment 4. The fish were kept in an aquarium with a temperature of 16° C and also showed a salt water preference. A rise in temperature of 1° and 2° did not affect this preference (see table 6).

The conclusion from these experiments is that it can be safely assumed that a difference in temperature of 5° C or less during a period of not more than 5 hours per day does not have any influence on the salinity preference of the fish. Therefore, the fact that in some experiments it was impossible to test the fish at the same temperature as the one at which they were kept during the experimental pretreatment cannot have influenced the results of the tests.

In Chapter IX it will be shown that at appropriate times in the physiological cycle of the animals a change in temperature to which the fish are exposed for more than 24 hours is able to induce a change in salinity preference.

2. THE EFFECT ON SALINITY PREFERENCE OF DIFFERENT DEGREES OF SALINITY IN THE TWO SALT WATER COMPARTMENTS

In Chapter II it was shown that during one double test the salinity in the different compartments changed in the course of the test, due to mixing of the water by the fish. Though in all cases the salinity on the salt water side remained higher than on the fresh water side (fig. 11) there is a possibility that the salinity preference may have become less pronounced by this mixing.

That the gradually decreasing difference in salinity between the salt and fresh water halves of the troughs cannot have had an important influence on the salinity preference is shown in the many experiments described in the following chapters in which the fish showed a consistent preference in all three sub-tests of one test.

The following experiments were made with the special aim of determining the effect on salinity preference of different degrees of salinity used in the two salt water compartments.

Experiment 5. In this case animals were used that showed a salt water preference when tested on three successive days in a trough containing 100 % and 50 % sea water. When tested again during the next three days in a trough containing 50 % and 25 % sea water they maintained their salt water preference (see table 7).

Experiment 6. This experiment was made with animals that showed a salt water preference when tested in 50 % and 25 % sea water. When tested in 100 % and 25 % sea water they maintained their preference for salt water (see table 7).

Experiment 7. The animals preferred fresh water when tested in 37.5 % and 25 % sea water. When tested subsequently in 50 % and 25 % sea water their fresh water preference remained (see table 7).

Experiment 8. In this case the fish preferred fresh water when tested in 25 % and 12.5 % sea water and maintained this preference when tested afterwards in 37.5 % and 12.5 % sea water (see table 7).

The conclusion from these experiments is in the first place that the salinity preference is not influenced by the degree of salinity used in the tests. In the second place it is evident that the animals make a choice between the relative degrees of salinity. Therefore, the gradual mixing of the fresh and salt water during a test cannot have affected their preference.

In table 7 the degree of responsiveness to the different salinities is also given. To this end the number of positions in each half of the trough obtained in the tests carried out on successive days with one and the same salinity were added and the percentage of positions calculated.

In experiments 5 and 6 it seems that the greater the difference between the salinities of both halves of the trough the more pronounced the salinity preference is. For in both experiments the percentage of positions in the salt water halves is larger when 100 % sea water was used. However, in both experiments the P values are far below the 0.01 level and, therefore, the above effect cannot have influenced the conclusions drawn from the preference tests.

In experiment 8 there is no difference in the responses to the various salinities, but in experiment 7 it seems as if the fresh water response is more pronounced when the difference in the salinities between both halves of the trough is smaller. This effect cannot be explained and more experiments are needed before a conclusion on this subject can be made.

The general conclusion that can be drawn from these experiments is that the degree of salinity used is not important as long as the difference between the salinities in both halves of the trough is great enough to allow the animals to make a choice. In the majority of experiments in the investigation concentrations have been used of

TABLE 7

The influence on salinity preference of the salt concentration in the two salt water compartments of the trough. For further explanation see table 6.

No of experiment	% sea water in compartment 1	% sea water in compartment 2	Preference: P for fresh water			P no preference	P for salt water			Percentage of positions in fresh water part of trough	Percentage of positions in salt water part of trough
5	100	50					⩽ 0.01	⩽ 0.01	⩽ 0.01		
	100	50					⩽ 0.01	⩽ 0.01	⩽ 0.01	29.7	70.3
	100	50					⩽ 0.01	⩽ 0.01	⩽ 0.01		
	50	25					⩽ 0.01	⩽ 0.01	⩽ 0.01		
	50	25					⩽ 0.01	⩽ 0.01	0.05–0.01	35.3	64.7
	50	25					⩽ 0.01	⩽ 0.01	⩽ 0.01		
6	50	25					⩽ 0.01	⩽ 0.01	⩽ 0.01	29.9	70.1
	50	25					⩽ 0.01	⩽ 0.01	0.05–0.01		
	100	25					⩽ 0.01	⩽ 0.01	⩽ 0.01	24.8	75.2
	100	25					⩽ 0.01	⩽ 0.01	⩽ 0.01		
7	37.5	25	⩽ 0.01	⩽ 0.01	⩽ 0.01						
	37.5	25	⩽ 0.01	⩽ 0.01		0.082				74.3	25.7
	37.5	25	⩽ 0.01	⩽ 0.01	⩽ 0.01						
	50	25	⩽ 0.01	⩽ 0.01	⩽ 0.01					64.9	35.1
	50	25	⩽ 0.01	⩽ 0.01		0,121					
8	25	12.5	⩽ 0.01	⩽ 0.01	0.05–0.01					63.8	36.2
	37.5	12.5	⩽ 0.01	⩽ 0.01	⩽ 0.01					63.1	36.9

3/8 and 2/8 sea water, i.e. 37.5 and 25 % sea water respectively and on the ground of the above results it can be safely assumed that these concentrations were appropriate for salinity tests.

3. THE MAINTENANCE OF FISH IN FRESH AND SALT WATER AND ITS INFLUENCE ON SALINITY PREFERENCE

Since for practical reasons it is so much easier to keep Sticklebacks in fresh water than in sea water, the majority of experiments were made with animals kept in fresh water. The results obtained in experiments described in the following chapters show that fish kept in fresh water prefer either fresh or salt water depending on their physiological condition at the time of the tests. This means that maintenance in fresh water does not prevent animals from preferring salt water. The following two experiments were made in order to compare the salinity preference of animals that were in the same physiological condition but were kept either in salt or in fresh water.

Experiment 9. The fish used in this experiment were caught in April on the sea-side of a sluice while migrating to fresh water. They were transported to the laboratory in salt water and tested immediately after arrival. Notwithstanding the fact that the animals had been living in sea water during at least 4 months they showed a preference for fresh water (see table 8).

After the tests they were transferred to fresh water and tested again after 24 and 48 hours. In table 8 it can be seen that their preference remained the same.

Experiment 10. Animals were used that had passed their reproductive period in fresh water and had subsequently been transferred to sea water. The controls remained in fresh water. Table 8 shows that both groups preferred salt water.

The conclusion from these experiments is that maintenance in either fresh or salt water does not influence the salinity preference of the animals. Therefore, the fact that the Sticklebacks were kept in fresh water in the majority of experiments cannot have influenced their salinity preference.

TABLE 8

The influence on salinity preference of the salinity of the maintenance medium. The vertical groups of three P-values represent the preferences in the three sub-tests of each test.

No of experiment	*Gonadal condition of fish*	*Preference of fish kept in fresh water*			*Preference of fish kept in salt water*			*Preference of fish previously kept in salt water 24 h. after transfer to fresh water*			*Preference of fish previously kept in salt water 48 h. after transfer to fresh water*		
		P fresh	*P no pref.*	*P salt*	*P fresh*	*P no pref.*	*P salt*	*P fresh*	*P no pref.*	*P salt*	*P fresh*	*P no pref.*	*P salt*
9	phase 2	no	tests	made	⩽ 0.01				0.85		⩽ 0.01		
					⩽ 0.01				0.08		⩽ 0.01		
						0,72		⩽ 0.01			⩽ 0.01		
					⩽ 0.01			⩽ 0.01			0.05–0.01		
					⩽ 0.01			⩽ 0.01			⩽ 0.01		
					⩽ 0.01			⩽ 0.01			⩽ 0.01		
10	phase 1a			⩽ 0.01			⩽ 0.01	no	tests	made	no	tests	made
				⩽ 0.01			⩽ 0.01						
				⩽ 0.01			⩽ 0.01						
				⩽ 0.01			⩽ 0.01						
				⩽ 0.01			⩽ 0.01						
				0.05–0.01			⩽ 0.01						
				⩽ 0.01			⩽ 0.01						
				⩽ 0.01			⩽ 0.01						
				⩽ 0.01		0,09							

4. THE SALINITY PREFERENCE OF THE DIFFERENT RACES OF STICKLEBACKS

In Chapter II it was mentioned that the Sticklebacks used in the salinity experiments belonged to three different races, namely the formae *trachura*, *gymnura*, and *semi-armata*. KOCH and HEUTS (1943) have shown that the *trachura* have a different salinity tolerance than the *gymnura*. The *semi-armata* belong physiologically to either the *trachura* or the *gymnura*.

The *trachura* can stand a salinity range from fresh water up to 45 pro mille without changing the concentration of their body fluids, whereas the *gymnura* have a corresponding range up to 40 pro mille. However, in the breeding season the capacity of osmotic regulation of both races decreases and the salinity tolerance decreases accordingly. The *trachura* are able to keep the concentration of their body fluids constant up to 22 pro mille, while the corresponding figure for the *gymnura* is about 10 pro mille. Further, HEUTS (1945) was able to demonstrate that the osmotic regulation is strongly dependent upon the temperature of the water. In both races the capacity of osmotic regulation decreases with increasing temperature.

Therefore, in using Sticklebacks of different races these physiological properties had to be taken into account. The first step to avoid interference of different capacities of osmotic regulation in salinity tests was to use salt water concentrations that could be tolerated equally well by both the *gymnura* and the *trachura*. It is clear that the concentrations used in the majority of experiments, namely about 12 and 8 pro mille, was suited for this purpose. This could also be demonstrated in the following experiments in which animals belonging to the three races were kept under similar external conditions and tested separately.

Experiment 11. The animals were under-yearling fish, measuring 3·5–4·5 cm when caught in fresh water in November 1955. Out of this group one lot of 5 *gymnura*, one of 5 *trachura* and one of 4 *semi-armata* were selected. In table 9 it can be seen that all three groups showed a consistent salt water preference.

Experiment 12. In this case the fish used were maintained under 16 hours day length and 20° C. They were under-yearling fish that had initially preferred salt water like those in the previous experiment. After a certain period of time this salt water preference changed to a fresh water preference. In the salinity tests one lot of 5 *trachura* and one of 5 *gymnura* were tested separately and the results are given in table 9. It can be seen that both races showed the same salinity preference.

It can be concluded from these experiments that the results of the salinity tests have not been influenced by physiological differences between the three races used.

TABLE 9

The salinity preference of the three races of Sticklebacks when exposed to similar external conditions. For further explanation see table 6

No of experiment	*Race*	*P for fresh water*			*Preference P no preference*	*P for salt water*		
11	*trachura*					⩽ 0.01	⩽ 0.01	0.05–0.01
						⩽ 0.01	⩽ 0.01	⩽ 0.01
	gymnura					⩽ 0.01	⩽ 0.01	⩽ 0.01
						⩽ 0.01	⩽ 0.01	⩽ 0.01
	semi-armata				0,068	⩽ 0.01	⩽ 0.01	
						⩽ 0.01	⩽ 0.01	⩽ 0.01
12	*trachura*	⩽ 0.01	⩽ 0.01		0,090			
		⩽ 0.01	⩽ 0.01	0.05–0.01				
	gymnura	⩽ 0.01	⩽ 0.01	⩽ 0.01				
		⩽ 0.01	⩽ 0.01	⩽ 0.01				

IV. THE ANNUAL CYCLE OF SALINITY PREFERENCE UNDER NATURAL CONDITIONS

In order to test the choice and value of the salinity preference method for experimental work on migration it was first of all necessary to make a series of tests during one year with animals coming from natural habitats. Since it was not always possible to catch the fish in their natural habitat in a number of cases fish had to be used that had been kept in the laboratory under conditions almost similar to those in nature. One series of experiments was made with adult fish and one with under-yearling animals. The results are given in fig. 12.

Experiment 13. In this case both male and female adult animals were used. In experiments 13 A and 13 F (see fig. 12) fish were used that had been caught in their natural habitat and were tested immediately after arrival in the laboratory. Those in 13 A had been caught in March on entering sluices while migrating to fresh water. The fish in 13 F had been caught in the sea on December 16, 1955.

The animals in groups 13 B, C, D, and E (fig. 12) had been caught in brackish water in March, 1953, while migrating to fresh water and were kept in the aquarium-house where conditions were almost similar to those in nature at that time of the year. However, the temperature was usually somewhat higher than in nature (see fig. 1).

The results of this series of experiments show that in the early months of the year until the beginning of the breeding season the adult fish prefer fresh water. At the end of the breeding season (in the second half of June or in the beginning of July) the animals prefer salt water.

The change from salt to fresh water preference in nature most probably takes place in the first month of the year, for in December the fish already prefer fresh water in some sub-tests, though the Pt value is still below the 0.01 level for salt. However, in Chapter IX it will be shown that at this time of the year the salinity preference depends very much on the temperature of the water and, therefore, the change from salt to fresh water preference may vary in time according to the water temperature.

The change from fresh to salt water preference seems to take place immediately after the termination of the breeding season. This could be confirmed in later experiments (Chapter V).

Experiment 14. Here under-yearling fish were used. Experiments 14 A and 14 F (see fig.12) were carried out with young fish caught in fresh water in March and November, 1955, respectively. They were tested immediately after arrival in the laboratory. The fish in experiments 14 B, C, D, and E had hatched in the laboratory in 1953 and were afterwards maintained in the aquarium-house where conditions were almost similar to those in nature (see fig. 1).

The result of this series of experiments shows that young fish prefer salt water from about two moths after hatching (probably still earlier) until at least December. In the early months of the year they prefer fresh water.

The changes in salinity preference shown in the above two experiments are in full agreement with the observations on migration made in nature (see Chapter II). Spring migration most probably starts in January or somewhat later, depending on the water temperature (see Chapter IX) and lasts until April, while autumn migration most probably starts shortly after the end of the breeding period. Young animals will be able to carry out seaward migration, for they already show a salt water preference two months after hatching.

It is not known, however, at what time these fish actually start to migrate to the sea. Some of the young fish seem to remain in fresh water, while others reach the sea. The young fish of experiment 14 F

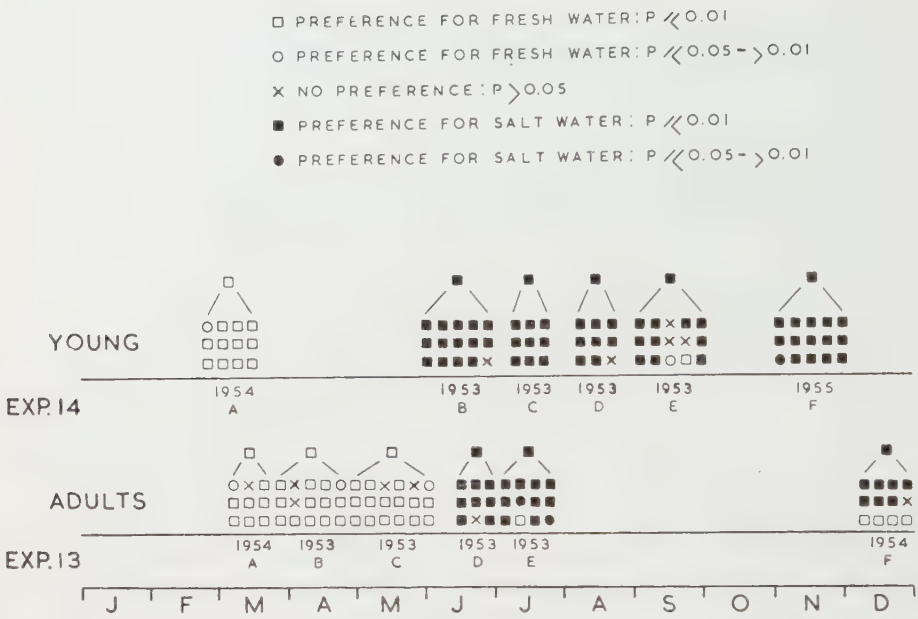

Fig. 12. Annual cycle of salinity preference of adult and under-yearling fish living under natural conditions. For further explanation of the figure see p. 225.

were caught in fresh water at a time when all adult fish had left the area. Though the former preferred salt water it must be presumed that they did not migrate seaward, for in December and January young animals were also caught at the same place as those in November.

The conclusion from the above experiments is that spring migration in the Stickleback is connected with a fresh water preference, whereas autumn migration is associated with a preference for salt water. The times at which the changes in salinity preference occur coincide with the onset of either spring or autumn migration. This justifies the choice of changes in salinity preference as criterion for the induction of migration-disposition. It is also clear that the method of testing salinity preference is very useful and valuable for giving information on the timing of migration.

V. GONADAL CYCLE AND SALINITY PREFERENCE

Since Rowan (see his review, 1938) concluded on the basis of experiments on migration in birds that the gonads were responsible for the onset of migration the attention of many investigators has been directed at this mechanism. The hypothesis that spring migration might be

caused by the developing gonads and autumn migration by gonadal regression is still rather widely held, though some investigators have objected to this assumption (see Chapter VI).

Let us first critically consider the above hypothesis. It is a fact that migration is closely correlated with the gonadal cycle. This has become evident from observations in nature and from experiments (see discussion at the end of this chapter). However, this does not imply a priori that migration is *caused* by gonadal changes. It is known that the gonadal cycle depends on the activity of the pituitary gland (see Part I, Chapter XI). It is also known that at the time of migration still another gland is hyperactive, namely the thyroid (see Chapter VIII). This means that at the time of migration the pituitary not only secretes increased amounts of gonadotrophic hormones, but also of thyrotrophic hormones, while it is possible that the production of still other trophic hormones may also show a maximum around the onset of migration. Therefore, it is not justified to assume a priori that migration is *caused* by gonadal hormones and not by any of the other hormones. Moreover, it is also possible that other mechanisms than the endocrine system may be responsible for the initiation of migration.

In the present chapter a number of experiments will be described which show that in most cases there is a close correlation between the gonadal cycle and changes in salinity preference. The possible *causal* relation between these two phenomena will be dealt with in the next chapter.

The salinity preference was determined in Sticklebacks that had been exposed to various external conditions when the gonads were in different phases of development. These experiments were made in connection with some of the experiments on the timing of the breeding season that have been described in Part I of the paper. Performance of the latter experiments also gave the opportunity to make salinity tests in the same animals. This procedure meant that the external conditions used in the present experiments depended on those used in Part I.

1. EXPERIMENTS AT 16 HOURS DAY LENGTH COMBINED WITH A TEMPERATURE OF 20° C

Experiment 15. The animals used were under-yearling fish that had hatched in the laboratory in April and May, 1953 and had subsequently been reared in the aquarium-house under almost natural conditions (see fig. 1). When these fish were tested in June, July and August they preferred salt water as was expected (see fig. 13). On October 1 they were transferred to a day length of 16 hours and a

temperature of 20° C. When tested in the second half of October and the first half of November the preference had changed to a fresh water preference. All five fish used in the experiment matured within 51 days of the transfer (mean duration of the response-time, 49 days).

This experiment shows that a fresh water preference can be induced at an abnormal time in the season by the same external condition that causes maturation of the gonads. In this case, therefore, a fresh water preference is closely correlated with the attainment of maturity.

Experiment 16. The animals in this experiment were the same as those of experiment 28, Part I. For details on the animals and conditions used the reader is referred to the latter experiment, but the main points will be shortly recalled here.

The animals were under-yearling fish that had been exposed to 16 hours day length and 20° C on July 19, 1954. From August 28 until October 6 they were subjected to 16 hours day length combined with a low temperature (4°). On the latter date they were transferred back to 20° while the day length remained 16 hours. The results of the salinity tests in these animals is given in fig. 13.

It can be seen that in August, (i.e. before exposure to the low temperature) the fish showed a salt water preference (Pt below the 0.01 level), though in most of the sub-tests there was no preference. In October, i.e. after the return to 20°, the preference gradually changed to one for fresh water, while in the second half of November the fish showed a consistent fresh water preference. The first fish attained maturity 31 days after the last transfer and the remaining four animals matured subsequently during November, December and January. The mean duration of the response-time of the five fish was 61 days after the transfer on October 6.

This experiment shows again that a fresh water preference can be induced at an abnormal time of the year by the same external conditions that induce gonadal maturation. This means that fresh water preference is closely correlated with the attainment of maturity. The reader will find more examples of this kind in experiments described in the following sections and chapters.

In Part I, Chapter VIII, it was shown that maturation can be prevented in under-yearling fish that are exposed to 16 hours day length combined with a temperature of 20° C when they are in phase 0 of gonadal development. Now it would be interesting to test the salinity preference of these animals.

Experiment 17. The animals used were the same as those described in experiment 24, Part I. They came from the same group as those in

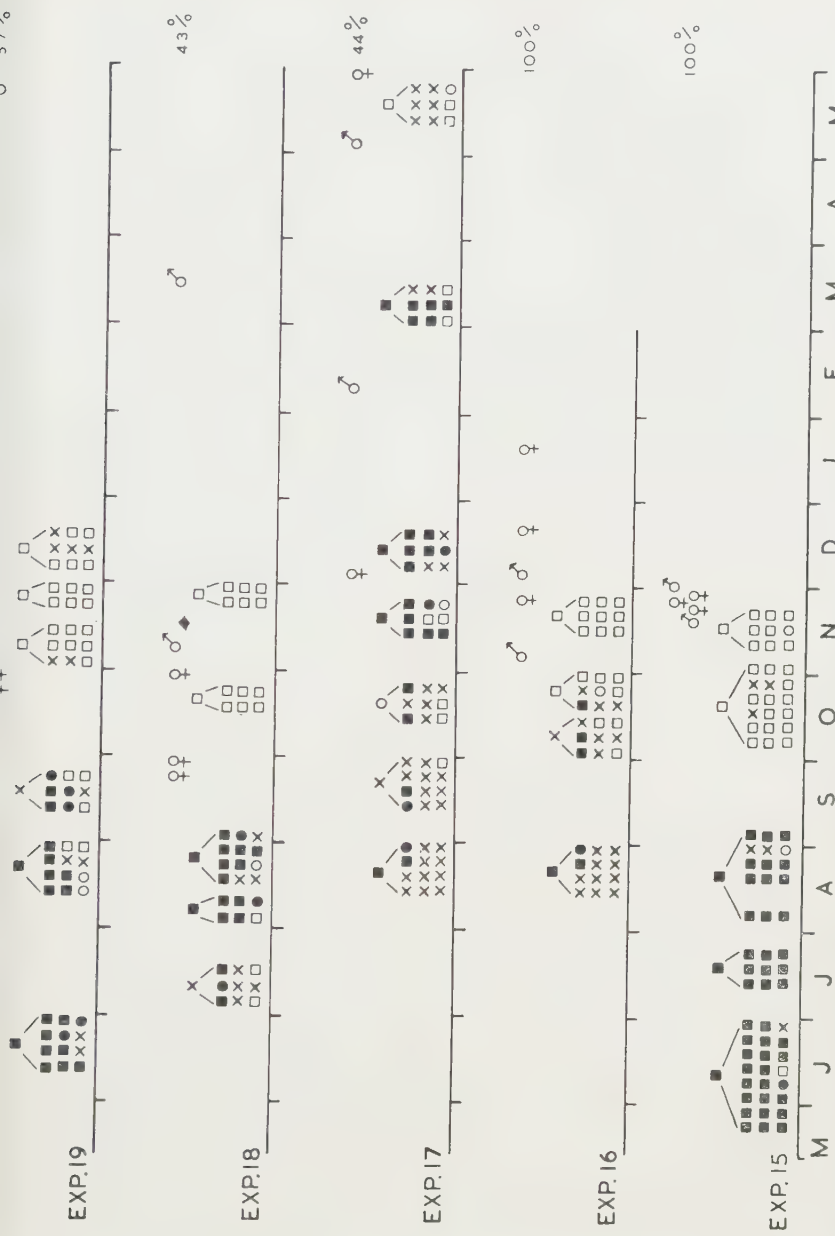

Fig. 13. Salinity preference of fish exposed to 16 hours day length and 20° C in different phases of gonadal development. The percentages indicate how many of the fish used matured; the date of maturation of each fish is indicated by the symbols ♂ and ♀. For further information see text and fig. 12.

the previous experiment but, unlike the latter, they were not subjected to the period of low temperature. In experiment 24, Part I, it was shown that some of the animals never attained maturity (see fig. 4,

July 19, 1954). The result of the salinity tests is given in fig. 13.

It can be seen that until November the animals showed no consistent salinity preference, though the Pt values indicated a salt water preference in August, no preference at the end of September, a weak fresh water preference at the end of October and a salt water preference in November. In the first half of December the first animal out of the lot of 9 matured, but when the remaining fish were tested in December, they still showed a prevailing salt water preference. In February of the next year a second fish matured, but the salinity tests in March still showed a preference for salt water. However, in May the preference changed to a prevailing fresh water preference. At that time 7 animals were left and two of them matured in May. None of the remaining 5 animals attained maturity within the next two months when the experiment was terminated.

This result shows in the first place that a delay in maturation (when compared with the time of maturation in the previous experiment made with fish of the same group) is closely correlated with a delay in the change in salinity preference from salt to fresh water. In the previous experiment the change in preference took place in November, whereas in the present experiment this change occurred in May. The same holds for maturation.

In the second place the experiment shows that the change in salinity preference from salt to fresh water is not caused by exposure during a certain period of time to a long day length and a high temperature, irrespective of whether the gonads matured during that time or not. This can be derived from the following data. In experiment 15 the animals matured on the average about one month after exposure to 16 hours day length and 20°, while the change in salinity preference took place after about half a month. In experiment 16 the corresponding figures were 5–6 and 3–4 months respectively. In experiment 17 the figures were 8–9 months (only some of the fish matured) and 10 months respectively. This means that the change in salinity preference from salt to fresh water is not due to exposure for a certain period of time to a long day length and a high temperature, for in all cases the duration of this exposure was different. It must be concluded that the change in salinity preference is correlated with gonadal maturation, for in the above three cases there was a difference of about one to two months between the mean date of maturity and the preference change, no matter how long it took the animals to attain maturity.

In the third place it has been shown here that the change in preference from salt to fresh water can take place in the absence of gonadal maturation, at least in some animals. This might indicate that the change in preference is not caused by the development of the gonads.

However, it is possible that in the fish which did not mature some gonadal development might still have taken place and it is suggested that the preference change might have been correlated with this slight development. Nevertheless, it is evident that attainment of maturity is not necessary for the preference change from salt to fresh water to take place. This is in agreement with the fact that in nature this change in preference takes place long before the fish are mature.

The next two experiments will give more evidence that the change in preference from salt to fresh water can take place in the absence of the attainment of full maturity, though it may still be associated with a slight gonadal development.

Experiment 18. The animals used here hatched in the laboratory in April and May, 1953 and were reared in the aquarium-house under conditions that were almost similar to those in nature (see fig. 1). On June 21 they were transferred to 16 hours day length and 20° C. The animals are identical with those described in experiment 24, Part I, fig. 4. When tested in July and August (see fig. 13) they showed a prevailing salt water preference (see Pt values). At the end of September, two out of the 12 fish matured. When tested in October the remaining 10 animals showed a consistent fresh water preference and within the following two months two of them matured. The tests carried out at the end of November with the remaining fish showed a fresh water preference. However, only one of the 8 animals matured (in March of the next year), whereas the remaining 7 fish did not attain maturity before June when the experiment was terminated.

Experiment 19. The animals used were under-yearling fish that had hatched in April and May, 1954, and were subjected to 16 hours day length and 20° C on June 14. The experiment with this group of fish has already been described in detail under experiment 24, Part I. The results of the salinity tests of this group are given in fig. 13.

In June and August the animals showed a prevailing salt water preference. In September the first signs of a preference change appeared (Pt value indicates no preference). In the second half of August one fish had matured and at the end of October two more animals attained maturity. The remaining four animals were tested in November and December and preferred fresh water, though none of these fish matured until May of the next year when one of them became ripe. In June the experiment was discontinued and at that time three animals were still immature.

These two experiments show again that a change in preference from

salt to fresh water can also take place in animals that do not attain maturity under 16 hours day length and 20° C. However, the experiments do not give conclusive evidence that the change in preference is not caused by the gonads, for it is possible that some gonadal development may have taken place, though the fish did not attain maturity. Other kinds of experiments are needed to determine whether the change in salinity preference is caused by the developing gonads or whether there is only a correlation and no causal relation between the two phenomena.

The general conclusion that can be drawn from the experiments described in this section is that attainment of maturity is always correlated with a change in preference from salt to fresh water. However, the latter change can also take place in the absence of complete maturation. The same external conditions that are able to induce maturation are also responsible for the change in salinity preference from salt to fresh water. However, in none of the experiments is there any evidence that the change in preference is *caused* by gonadal hormones, since it is known that the activity of the pituitary in secreting gonadotrophic and possibly other trophic hormones has been stimulated simultaneously with the secretion of gonadal hormones. Besides, the change might be due to a different mechanism than the endocrine system.

2. EXPERIMENTS AT 8 HOURS DAY LENGTH COMBINED WITH A TEMPERATURE OF 20° C

In Part I, Chapter VI, it was shown that when animals are exposed to 8 hours day length and 20° C in phase 1 of gonadal development maturation is inhibited, though some gonadal development may still take place. The following experiment was done in order to determine the salinity preference of animals kept under the above condition.

Exberiment 20. The animals used were under-yearling fish that had hatched in April and May, 1954 and had been reared in the aquarium-house under almost natural conditions (see fig. 1). On June 14 they were exposed to 8 hours day length and 20° C. An experiment with fish of the same group that were kept under 16 hours day length and 20° C has been described in experiment 19. The result of the present experiment is given in fig. 14.

It can be seen that nowhere in the period of 14 months during which the animals were kept under the same condition did the fish show a prevailing fresh water preference, though they did in a small number

of sub-tests. The Pt values were below the 0.01 level in 11 out of the 13 test-series and indicated no preference in two cases. None of the animals attained maturity during this time.

The conclusion is that under conditions inhibiting the attainment of maturity the change in preference from salt to fresh water is also inhibited. The experiment again shows that maturation and the change in preference from salt to fresh water are closely correlated.

This correlation was stressed once again when on July 20, i.e. after being for about 14 months under 8 hours day length and 20° C, four of the animals were transferred to 16 hours day length and 20° C. The four fish all attained maturity and the mean response-time was 26 days after the transfer. Fig. 14 shows that during this time the salt water preference also changed to a fresh water preference.

The reader will find another example of a long lasting inhibition of maturation which is correlated with a persistent salt water preference in experiment 28, fig. 16. This experiment will be dealt with in another connection.

The fact that under the above condition no change in salinity preference takes place shows that no intrinsic mechanism is able to induce such a change. The change in preference can only take place under certain external conditions. The same reasoning held for the attainment of maturity (see p. 153).

Experiment 21. In this case under-yearling fish were used that had hatched in April and May, 1954 and had been reared in the aquarium-house under almost natural conditions (see fig. 1). On July 19 they were exposed to a day length of 8 hours and a temperature of 20° C. An experiment with animals of the same group that were exposed to 16 hours day length and 20° C on the same day has been described under experiment 17. The result of the present experiment is given in fig. 14.

During August, September and October the animals showed a prevailing salt water preference, though the Pt value in September did not indicate any preference. However, in November the fish changed their salt water preference to one for fresh water, though not very convincingly since there was no preference for either salt or fresh water in the majority of sub-tests. In December the same tendency towards a fresh water preference was present, though less marked than in November. In March the Pt value did not indicate any preference and in May the value of Pt was again below the 0.01 level for fresh water, though there was no preference in some sub-tests. During this period of time none of the fish matured, since they had evidently been in phase 1 of gonadal development at the beginning of the experiment (see table 5).

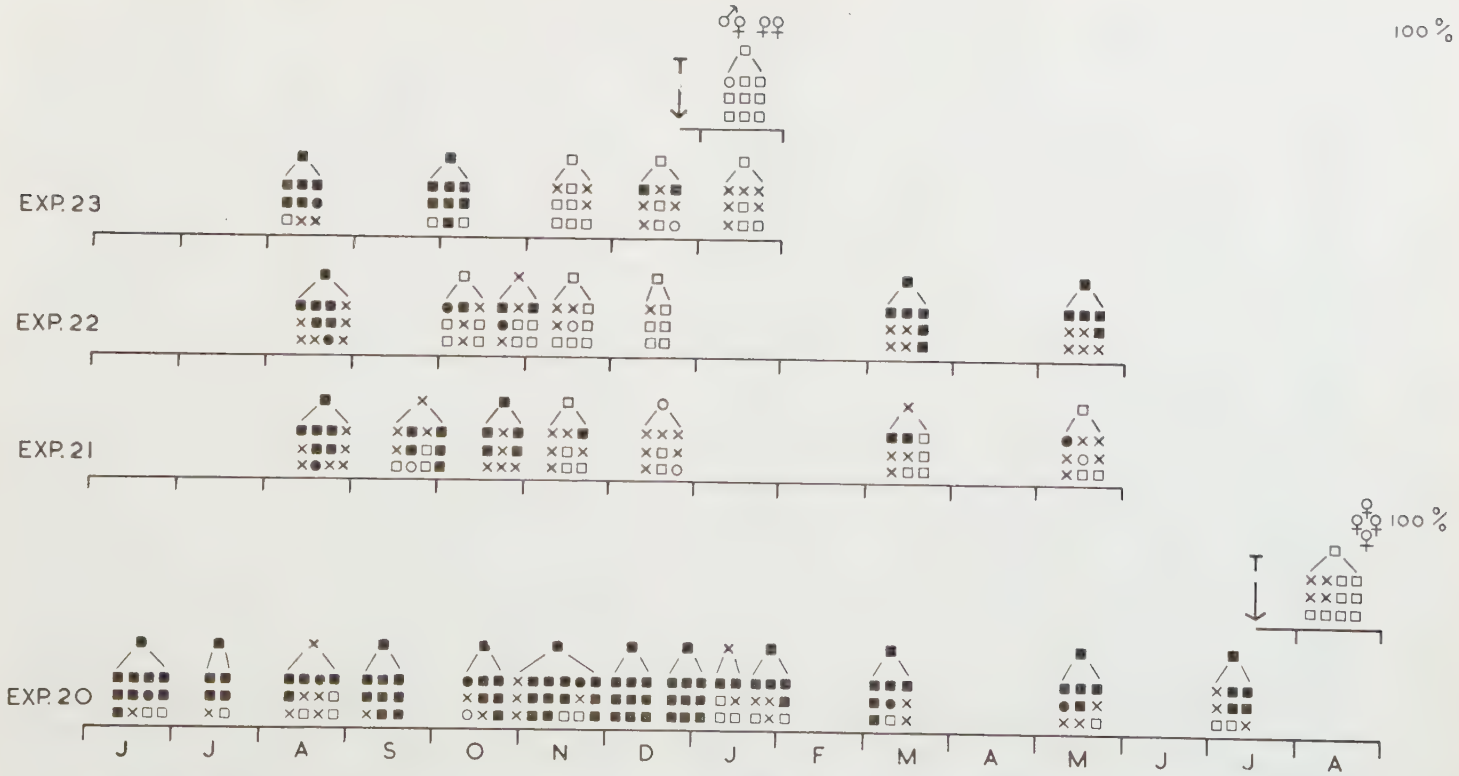

Fig. 14. Salinity preference of fish exposed to 8 hours day length and 20° C in different phases of gonadal development. T = date of transfer of fish to 16 hours day length and 20° C. For further explanation see figs. 12 and 13.

In this experiment it is shown again that a change in salinity preference from salt to fresh water need not be correlated with attainment of maturity. However, in Part I, Chapter VI, it has been shown that under these conditions some gonadal development still takes place. Therefore, it is suggested that the change in salinity preference (though it was not very convincing) shown in the present experiment may have been correlated with a slight gonadal development.

Experiment 22. The animals used came from the same group as those in the previous experiment and were also exposed to 8 hours day length and 20° C on July 19. However, the fish in the present experiment were exposed to a period of low temperature (4°) between August 28 and October 6. The day length remained 8 hours. On October 6 the temperature was 20° again. The experiment is described in detail in experiment 20, part I. In experiment 16 (Part II) animals were similarly kept under 16 hours day length and 20° C. It was shown there that the period of low temperature was favourable for gonadal development and it was supposed that at 8 hours day length this treatment would also be favourable. The result of the salinity tests is given in fig. 14.

In August the animals preferred salt water, but in October, i.e. shortly after the treatment with low temperature was ended, the fish showed a tendency towards a fresh water preference (Pt value below 0.01 for fresh), though it was not consistent in all sub-tests. At the end of October, in November and in December the main preference remained for fresh water, though not consistently in all sub-tests. In March and May, however, the animals again preferred salt water (Pt value below 0.01 for salt). During this time none of the animals matured, since they were evidently in phase 1b at the beginning of the period of low temperature (see table 5).

This experiment shows that a change in preference from salt to fresh water need not be correlated with the attainment of maturity. Nevertheless, it is not unlikely that under the above conditions some gonadal development may have taken place and the change in salinity preference would then have been correlated with this slight development. This suggestion is supported by the fact that in the present experiment the fresh water preference was more consistent than that in the previous one. This was most probably due to the period of low temperature which is known to have a favourable influence on gonadal development in phase 1a. The fish in the present experiment, however, were in phase 1b which cannot be stimulated to develop until maturity by the above condition, though some development may still have taken place.

The present experiment also shows that there was an alternation of salt and fresh water preference in the course of time. In Part I, Chapter VI, it was shown that under 8 hours day length and 20° C cyclical changes in gonadal development occur, though the gonads do not mature. It seems likely that the above changes in salinity preference are correlated with the gonadal cycle.

Experiment 23. The animals used were under-yearling fish that had hatched in April and May, 1954 and had been reared in the aquarium-house under conditions almost similar to those in nature (see fig. 1). On October 9 they were subjected to 8 hours day length and 20° C. The results of the salinity tests are given in fig. 14.

In August and October the fish mainly preferred salt water, but in November this preference had changed to one for fresh water, though it was not consistent in all sub-tests. This prevailing fresh water preference remained in December and January. The fish were kept under the same condition until May, but none of them matured.

On December 22 four of the animals were transferred to 16 hours day length and 20° C where they matured on the average 28 days after the transfer. When tested shortly before the attainment of maturity the animals showed a consistent fresh water preference.

This result shows again that a change in preference from salt to fresh water can take place in the absence of the attainment of maturity, though it is still possible that some gonadal development may have taken place.

The fact that in some experiments the animals did not show a change in preference from salt to fresh water under 8 hours day length and 20° C (experiments 20 and 28), whereas they did in others (experiments 21, 22, and 23) must be explained in the following way. The fish of experiments 20 and 28 had been exposed to the above external condition at a very early stage (phase 0 or beginning of 1a) of gonadal development or recrudescence respectively. The fish in experiments 21, 22, and 23 were subjected to this condition at later phases in gonadal development (end of phase 1a or in 1b). This is most probably the reason that in the latter the gonads developed a little further than in the former. The change in salinity preference is most probably correlated with this partial development.

The conclusion of the experiments described in this section is that a change in salinity preference can take place in animals that do not attain maturity. However, in case the animals do mature the change in salinity preference is more consistent than when no maturation takes place. This can be seen when the number of sub-tests in which a

fresh water preference was shown is compared in animals that did attain maturity and in those that did not (see fig. 13 and 14). It seems, therefore, as if there is a correlation between partial gonadal development and a rather weak fresh water preference, and between complete development (attainment of maturity) and a strong fresh water preference. However, none of the experiments is conclusive as to a causal relation between secretion of gonadal hormones and salinity preference. It has also been shown that the change in salinity preference from salt to fresh water is caused by particular external conditions and not by an intrinsic mechanism.

3. THE SALINITY PREFERENCE DURING LONG PERIODS OF CONSTANT EXTERNAL CONDITIONS

In Part I, Chapter X, it was demonstrated that animals kept under constant external conditions of 16 hours day length and 20° C showed alternating reproductive and non-reproductive periods. Animals with gonads in phase 2 are able to mature under 8 hours day length and 20° C, but when they are maintained afterwards under this condition for long periods of time they do not show alternating reproductive and non-reproductive periods. In the following experiments fish were tested that had been kept under either of the two conditions for long periods of time beginning shortly after the attainment of maturity.

An attempt was made to test animals that were in the sexual phase, but this was impossible in the majority of tests, because in this phase the fish are very aggressive and are thus prevented from making an actual choice. Therefore, most of the tests had to be discarded. However, in a few tests the fish were not aggressive for some reason and the results of these tests will be given below.

a. *The salinity preference during the reproductive period*

Experiment 24. The animals had built nests for the first time around April 15. They were kept under constant conditions of 16 hours day length and 20° C. Fig. 15 shows that during the following 4 weeks they showed no consistent preference for either salt or fresh water. The Pt values indicate an alternation in salinity preference.

Experiment 25. In this case one male was used that had a nest for about one month and two females that had spawned for the first time three days before the beginning of the experiment. They were kept under constant conditions of 16 hours day length and 20° C. Fig. 15 shows that also in this case there was no consistent fresh water preference, though the Pt values were below the 0.01 level for fresh.

The results from these experiments show that the consistent fresh water preference which animals have shortly before attainment of maturity may already begin to change during the reproductive period. An explanation of this phenomenon will be given on p. 264.

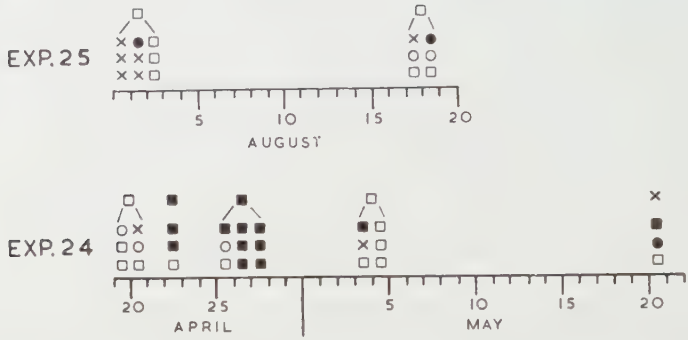

Fig. 15. Salinity preference of two groups of fully mature fish. For further explanation see fig. 12.

b. *The salinity preference during the non-reproductive period*

In Part I, Chapter IX, the criterion for determination of the end of the breeding period has been described. In the following experiments the animals were tested at various times in their non-reproductive phase. Groups of 2 to 4 animals that had advanced about equally far in the non-reproductive phase were tested. In fig. 16 the mean number of days the group had advanced in the latter phase is given.

i. The preference under 16 hours day length and 20° C

Experiment 26. Fig. 16 shows that during the first two months after the end of the breeding period the fishes showed a prevailing salt water preference, with an exception at 17 and 23 days, when the Pt value was below the 0.01 level for fresh or indicated no preference respectively. However, it can be seen that the preference in the sub-tests was far from consistent in these cases. About three months after the end of the breeding period the salt water preference started to change into a fresh water preference, while the latter was consistent about 4 months after the end of the breeding phase. During the experiment many animals that had been tested died, but the few that survived matured 150, 157, 158, and 200 days respectively after the end of the reproductive period (mean duration 166 days).

Experiment 27. A similar experiment was made with a group of four females that had ovulated a number of times during a period of about

two months (see experiment 37, Part I) and was tested at various times after the end of the reproductive phase. The results are given in fig. 16.

It can be seen that at the end of the breeding period the fish showed a preference for salt water, though this is only expressed by the Pt value, while the sub-tests did not show a consistent preference. Two months after the end of the breeding period the animals did not seem to prefer either salt or fresh water. But after about three months a definite change towards a fresh water preference appeared and after about 4 to 5 months this preference was consistent in all sub-tests. Three out of the four animals that were used matured after 160, 165, and 180 days respectively, mean duration of the response-time 168 days.

The results of these two experiments show that notwithstanding the external conditions had remained constant all the time, the preference changed from fresh to salt water after a certain period of time. In Part I, Chapters IX and X, a similar phenomenon has been demonstrated with respect to the termination of the breeding period. The change in salinity preference can be explained in the same way as the termination of the breeding phase, namely that an intrinsic factor must have been responsible.

The change in salinity preference from salt to fresh water that occurred after about 3 to 4 months can also be explained in the same way as the attainment of maturity after about the same time. It must be assumed that after a certain period of time the fish again became sensitive to the stimulating external condition which induced gonadal maturation and also the change in preference from salt to fresh water.

ii. The preference under 8 hours day length and 20° C

Experiment 28. The animals (only males) were exposed to 8 hours day length and 20° C in phase 2, which means that they attained maturity under this condition. Fig. 16 shows that after the breeding period had ended the animals preferred salt water. There was only one exception when the fish were tested 28 days after the end of the breeding phase. In this case the Pt value indicated no preference. However, in all other tests the fish showed a prevailing salt water preference and this did not change in the course of the period of 8 months during which the animals were maintained under this constant external condition. This is in agreement with the fact that none of the animals attained maturity during this time.

The facts that under this condition the animals had a shorter breeding period than under 16 hours day length and 20° C (45.8 days and 91.4 days respectively, see p. 182 and 178) and that they also started to prefer salt water earlier than the animals under 16 hours

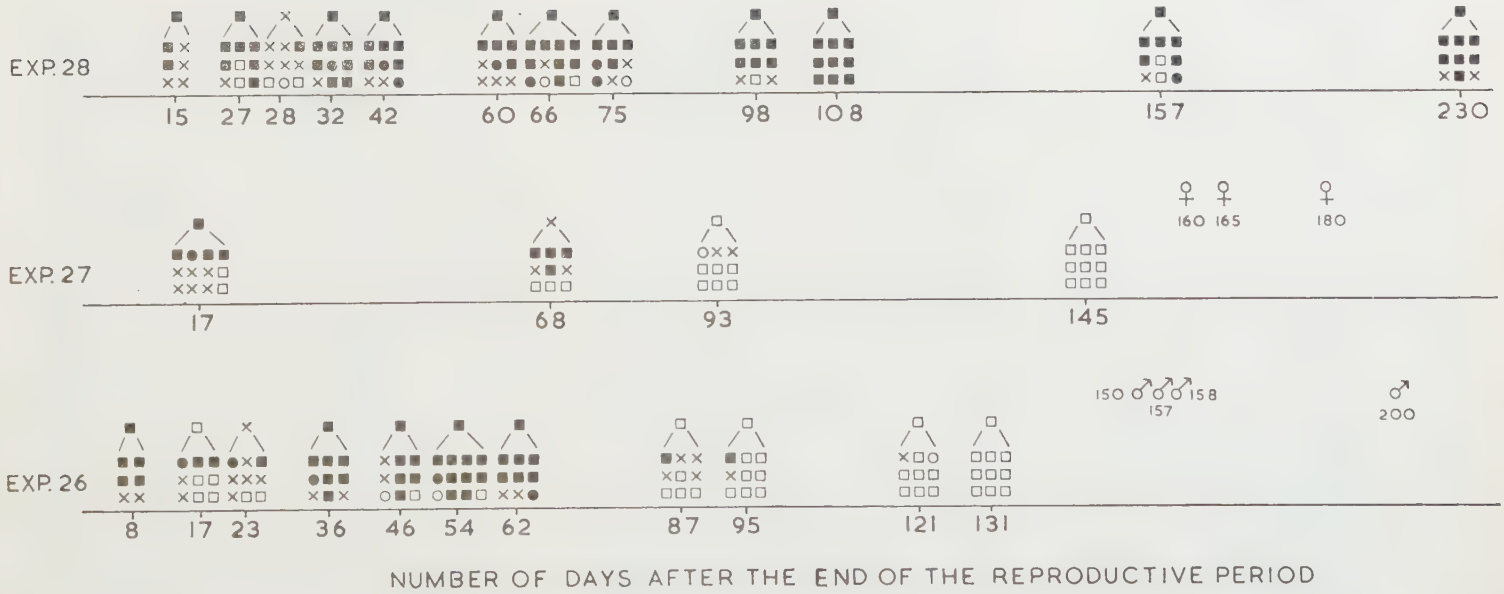

Fig. 16. Salinity preference of fish tested at various times after the end of the reproductive period. The fish of exp. 26 (♂♂) and 27 (♀♀) were exposed to 16 hours day length and 20° C; those of exp. 28 (♂♂) were subjected to 8 hours day length and 20° C. The symbols ♂ and ♀ with the accompanying figures indicate the date of attainment of maturity and the number of days between this date and the end of the previous breeding period. For further explanation see fig. 12.

day length can be explained in the same way. It is the external condition, which together with an intrinsic factor determines the duration of the breeding period (Part I, Chapter IX) and also the onset of the change in preference from fresh to salt water.

The experiment shows at the same time that there is no intrinsic factor which is able to induce the change in preference from salt to fresh water under the above conditions (see also p. 243).

The conclusions that can be drawn from the experiments described in this section are in the first place that after the attainment of maturity the fish no longer show a consistent fresh water preference as they did prior to this phase. However, the definite change in salinity preference from fresh to salt water takes place only after the breeding period has ended.

In the second place it is shown that the onset of this preference change is determined by the external condition in co-operation with an internal factor, in the same way as the end of the breeding phase is determined.

In the third place it is evident that under 8 hours day length and 20° C no intrinsic factor is able to induce the change in preference from salt to fresh water. The latter change depends on the presence of appropriate external conditions.

It is evident that the cycle of salinity preference is very closely correlated with the gonadal cycle, though none of the experiments is conclusive as to whether or not there is a *causal* relation between the two phenomena.

4. DISCUSSION

The general conclusion that can be drawn from the experiments described in the previous sections is that the change in preference from salt to fresh water is closely correlated with gonadal development and the change from fresh to salt water with gonadal regression. This is in full agreement with migration in nature.

Observations on migration in nature formed the initial basis for the hypothesis that migration is *caused* by either gonadal development or regression. These observations were supported by experiments of Rowan (1929, 1930, 1932, 1938, and 1946) who demonstrated that when the gonads of birds were stimulated by light treatment in the middle of winter the animals moved away when released. Moreover, recovery of a few marked birds showed that most of the treated animals had moved north, i.e. in the direction of spring migration. When the control animals were released some of them remained in the vicinity

of the releasing site, while some moved south (a kind of delayed autumn migration). It was evident, therefore, that a premature spring migration had been induced by the light treatment. WOLFSON (1942) was able to confirm the results of ROWAN's experiments.

However, the interpretation by ROWAN of the results, namely that the gonadal hormones are responsible for the induction of spring migration, was premature because his experiments were not conclusive on this point. For, as was already mentioned before, at the time that the gonads secrete increased amounts of sex hormones, the pituitary secretes increased amounts of gonadotrophic hormones and possibly also of other trophic hormones, e.g. thyrotrophin which induces an increased output of thyroid hormone (HÖHN, 1950). Besides, it is possible that factors other than hormonal might cause the onset of migration.

More direct experiments on the influence of gonadal hormones on migration have been made by SCHILDMACHER who used the restlessness of caged birds in autumn as indicator for the migration-drive. This restlessness is particularly obvious in birds that migrate at night. SCHILDMACHER (1933, 1934) found that in autumn the restlessness of female birds could be eliminated by injections of estrogens. SCHILDMACHER (1937, 1938) and PUTZIG (1937, 1938) were able to induce a precocious spring migration urge in winter by light treatment. However, GIERSBERG and STADIE (1934), and STADIE (1938) did not obtain conclusive results with injection of different hormones.

Though some of the results of the above experiments seem to indicate that gonadal hormones may have a direct effect on migration the experiments are not conclusive, since it is known that gonadal hormones may influence the secretion of trophic hormones by the pituitary (e.g. gonadotrophic and thyrotrophic hormones, see BURROWS, 1949, and BUDDENBROCK, 1950). Thus it cannot be concluded from the above experiments whether the effect of gonadal hormones on migration was direct or whether it was achieved by the mediation of other hormones.

Gonadal hormones have been shown to stimulate the locomotory activity in fishes (HOAR, MCKINNON and REDLICH, 1952; HOAR, KEENLEYSIDE and GOODALL, 1955). It is conceivable, according to the authors, that an increased general activity of fish during migration is favourable for the long journeys these animals have to make before reaching the place of destination. The same investigators were also able to show that thyroid hormone exerted a similar influence on the general activity of the fish as did the gonadal hormones. In mammals it is known that thyroxine stimulates general activity (BUDDENBROCK, 1950). However, the experiments of the above investigators are not conclusive as to whether the effect of gonadal hormones on the activity

of the fish was direct, or whether it was indirect by stimulating the secretion of thyrotrophin by the pituitary and thus of thyroid hormone. Another effect of gonadal and thyroid hormones was that under their influence the animals became more sensitive to external stimuli, a phenomenon that the authors believe might also play a rôle in migration.

The experiments with Sticklebacks so far have shown that there is a close correlation between the gonadal cycle and the cycle in salinity preference. It has been argued that on the ground of these experiments and also on those made in birds it is premature to conclude that gonadal hormones are responsible for the onset of migration. In the next chapter experiments will be described where gonadectomized animals were used in order to determine whether or not there is causal relation between the gonadal cycle and migration.

VI. THE SALINITY PREFERENCE IN GONADECTOMIZED FISH

Before the results of the experiments with gonadectomized fish will be described it is necessary to discuss briefly the effects that can be expected on the basis of the hypothesis that the gonadal cycle causes the onset of migration.

In the case of the Stickleback it is expected that gonadectomy in fishes preferring fresh water would result in a change in salinity preference from fresh to salt water. Further, it can be expected that it would be impossible to induce a fresh water preference in gonadectomized fish preferring salt water. The results of the following experiments will be regarded in the view of these concepts.

1. THE EFFECT OF GONADECTOMY ON ANIMALS PREFERRING FRESH WATER

Experiment 29. The animals used here had been artificially induced to mature in November and had been transferred to the aquarium-house (conditions almost natural) in January. On the ground of the observation that some of the fish stopped nestbuilding and lost their nuptial coloration at the end of January it was assumed that the breeding period was about to end. When the fish were tested at the end of January and the beginning of February they still showed a fresh water preference (see fig. 17), though this preference was far from consistent in the sub-tests.

On January 28 some of the fish were castrated (males only) and both the castrates and control fish were kept in the aquarium-house

under conditions almost similar to those in nature (see fig. 1).

a. *Controls*. Fig. 17 shows that at the end of February and in March the animals preferred salt water (non-reproductive phase). But in the first half of April the first signs of a change in salinity preference appeared and in the second half of that month the fish showed a consistent fresh water preference. This was in agreement with the fact that three out of the four animals used in the tests built a nest at that time.

b. *Experimentals*. Fig. 17 shows that when the animals were tested about one week after the operation they showed a fresh water preference slightly more pronounced than before the operation. They maintained this preference all through February and the first half of March. However, in the second half of the latter month the preference suddenly changed to a salt water preference. At the beginning of April most animals died and the experiment was ended.

When the fish were examined macroscopically they did not show any trace of regenerated testis tissue. The kidneys were very thin (they are heavily swollen at maturity) and none of the fish showed any trace of nuptial coloration.

The conclusion that can be drawn from this experiment is that gonadectomy did not result in a change in preference from fresh to salt water as would be expected. The change in salinity preference that took place 6 weeks after the operation cannot be regarded as an effect of the gonadectomy, for the change took place even earlier in the controls, namely about 4 weeks. This phenomenon will be discussed later (see p. 264).

Experiment 30. The fishes used came from the basin outside the laboratory and were transferred to the aquarium-house (conditions almost similar to those in nature) at the beginning of February, 1954. The animals were tested in the second half of February and showed a fresh water preference (see fig. 17, controls). The animals were castrated (males only) and remained in the aquarium-house together with the controls (for conditions see fig. 1).

a. *Controls*. In March the controls still preferred fresh water as can be seen in fig. 17, though not very consistently. Four out of the five animals used in the tests matured at the end of March and the beginning of April. At the end of the latter month the Pt value showed that the fish had no preference. The reader is reminded that often after the attainment of maturity the animals no longer show a pronounced fresh water preference (see fig. 15). The animals were tested again in the beginning of June at the time when they had just finished their reproductive period (no more nestbuilding; loss of nuptial coloration). They then showed a prevailing salt water preference, though not

consistent in all sub-tests. The same was true in July and August.

b. *Experimentals*. Fig. 17 shows that when the fish were tested within one week of the operation they still preferred fresh water, but about one week after that they suddenly showed an almost consistent salt water preference. This, however, did not last long and in the second half of March there was again a prevailing fresh water preference. This preference lasted all through the following months until at least the end of August. In October, November, and December the preference had changed to a preference for salt water.

On May 15 one of the gonadectomized animals built a nest. Autopsy revealed the presence of some testis tissue about 1 mm in diameter; the kidneys were enormously swollen. This animal had been used in the tests until the above date. Out of the five remaining animals one showed traces of nuptial coloration on June 17. Autopsy revealed a tiny piece of testis tissue of less than 1 mm diameter and the kidneys were moderately swollen. This fish had been used in the tests until then. Autopsy of the remaining four fish in December revealed no trace of gonadal regeneration, while the kidneys were very thin. These fish also had never shown any signs of nuptial coloration after the operation.

Since gonadal regeneration took place in only two out of the six animals used, it can be safely assumed that the fresh water preference that was shown by this group of fish had not been caused by the two fish with the regenerating gonads. Besides, when these two animals had been discarded the remaining fish retained their fresh water preference.

The conclusion from this experiment is that in this case a temporary change in salinity preference took place shortly after the operation, most probably as a result of the gonadectomy. However, shortly afterwards the initial fresh water preference returned and lasted during the following 6 months. The definite change in salinity preference took place after September, i.e. at least 5 months later than the controls. The latter effect will be discussed on p. 264, while on p. 265 an explanation will be given of the temporary change in salinity preference that took place shortly after the removal of the gonads. However, it is already stated here that this effect does not support the theory of Rowan, for then the return to the initial fresh water preference cannot be explained.

Experiment 31. This experiment was carried out with females that came out of the same group of animals as the males in the previous experiment. They were transferred to the aquarium-house in the second half of January. When tested at this time and in the first half of February

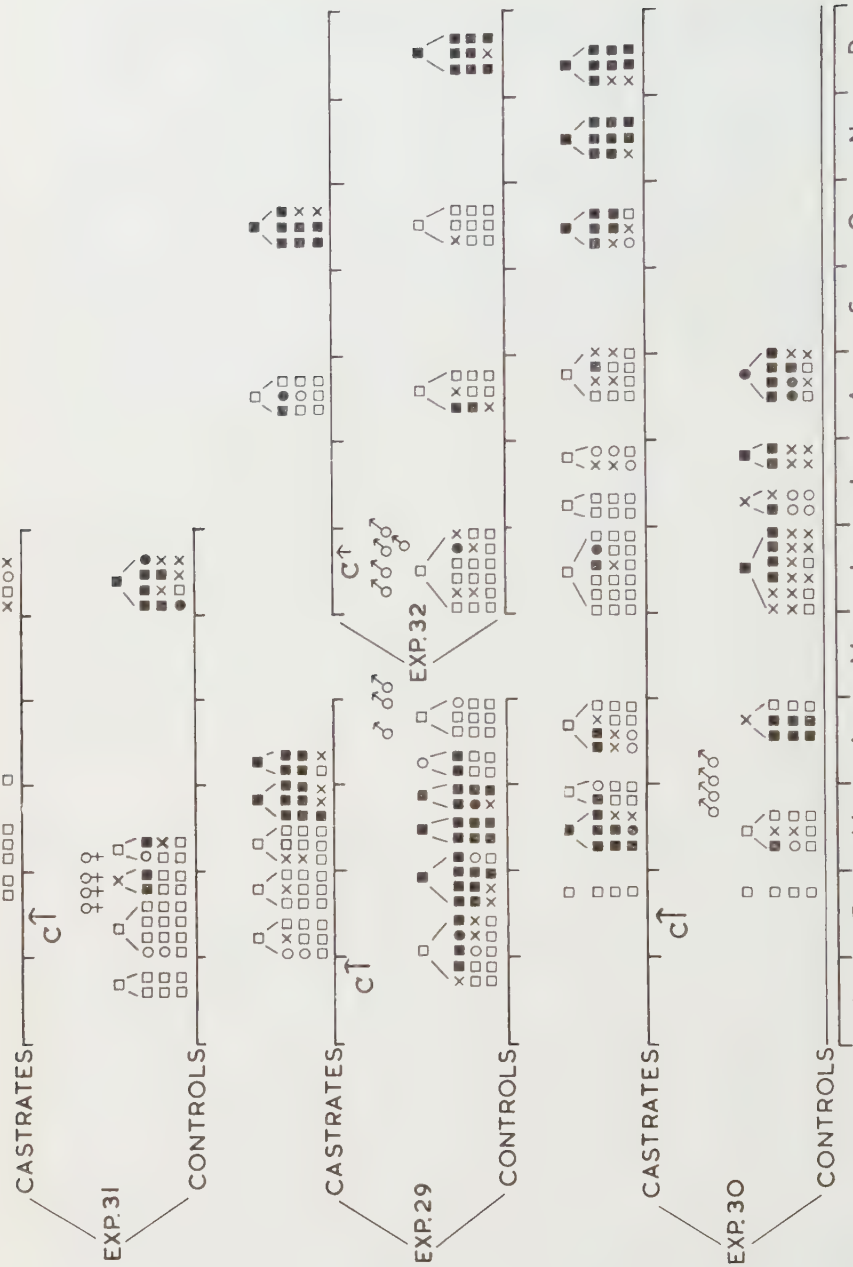

Fig. 17. Salinity preference of gonadectomized and normal fish initially preferring fresh water and kept under natural conditions from the date of castration (C). The symbols ♂ and ♀ indicate the dates of maturity of the controls. For further explanation see fig. 12.

the animals showed a consistent fresh water preference. The females were ovariotomized after the last test. Both control and experimental fish were kept in the aquarium-house under almost natural conditions (see fig. 1).

a. *Controls*. Fig. 17 shows that four out of the five controls matured at the end of February and the beginning of March. During that time they still showed a fresh water preference, though not consistently in all sub-tests. The animals were tested again in June and then had a prevailing preference for salt water.

b. *Experimentals*. Fig. 17 shows that shortly after the operation there was a slight tendency towards a salt water preference, though the Pt value was still below the 0.01 level for fresh water. The following tests made in March and April showed that the fish still preferred fresh water. In June there was a tendency towards a change in salinity preference, for the Pt value no longer indicated a preference for fresh water. At this time the experiment had to be terminated because most animals died shortly after the last tests.

The experimental group consisted of five animals and in one of them the ovaries had regenerated at the end of April. This fish had been used in the experiments until then. None of the remaining four animals showed any traces of gonadal regeneration when examined at the end of the experiment. Therefore, it is very unlikely that the one fish with regenerated gonads was responsible for the fresh water preference until April.

This experiment shows again that no lasting change in salinity preference takes place shortly after gonadectomy. There is evidence that in the controls the change in preference from fresh to salt water took place earlier than in the experimentals. This point will be discussed later on p. 264, while the temporary change shortly after the operation will be dealt with on p. 265.

Experiment 32. The animals had been kept in the basin outside the laboratory and were transferred to the aquarium-house in the first week of June. They immediately started to build nests. When tested they showed a prevailing fresh water preference (see fig. 17). Castration of the males took place in the last week of June. The experimental fish as well as the controls were kept in the aquarium-house under almost natural conditions (see fig. 1, 1954).

a. *Controls*. The fish showed a prevailing fresh water preference in August and October (see fig. 17). This was rather surprising since nestbuilding had already stopped at the end of August. It was noticed, however, that in these animals the nuptial coloration seemed to last for a longer period of time after breeding than usual. This might

perhaps account for the fresh water preference in October. No explanation for this longlasting fresh water preference can be given. When the fish were again tested in the first week of December the salinity preference had changed and the animals preferred salt water.

b. *Experimentals*. Fig. 17 shows that in August the fish still had a preference for fresh water, but that in October they preferred salt water. This is the only case in all the experiments with gonadectomized fish in which the operated animals started to prefer salt water earlier than the controls. This point will be discussed on p. 265.

Autopsy at the end of the experimental series revealed that in none of the five castrated fish was there any trace of gonadal tissue. This is in agreement with the fact that all animals had completely lost their nuptial coloration about two months after the operation.

The conclusion from this experiment is that in animals initially preferring fresh water gonadectomy does not result in a change in preference from fresh to salt water. A similar conclusion can be drawn from experiments 33 and 34, fig. 18, which will be described in the next section.

2. INDUCTION OF A FRESH WATER PREFERENCE IN GONADECTOMIZED FISH PREFERRING SALT WATER

In the introduction of this chapter it was mentioned that on the grounds of the hypothesis that the gonadal cycle is responsible for the causation of migration, it must be expected that it would not be possible to induce a fresh water preference in gonadectomized fish that preferred salt water. This assumption was tested in the following experiments.

The animals used in the next two experiments had been caught in fresh water in April, 1955 and were tested immediately after arrival in the laboratory. Fig. 18 shows that the fish preferred fresh water. After the tests some of the males were castrated and both experimental and control animals were kept in the aquarium-house under conditions almost similar to those in nature (see fig. 1).

Around May 15 the animals were tested again and fig. 18 shows that the castrated animals still preferred fresh water, though somewhat less pronounced than before the operation. There was no opportunity to test the controls also, but it can be safely assumed that they too would have preferred fresh water. So far the experiment is in agreement with the previous experiments in that gonadectomy in fishes preferring fresh water does not result in a change in preference from fresh to salt water.

With these animals two different experiments were started. In the

first experiment (33) an attempt was made to quickly induce a salt water preference after which the animals would be exposed to conditions known to be favourable for the attainment of breeding. By doing so it would be possible to show whether or not a change in salinity preference from salt to fresh water could be induced in gonadectomized animals. Experiment 34 was designed with a similar aim; only the external condition under which it was carried out was different.

Experiment 33. The above animals, both operated and control fish, were exposed on May 17 to a decrease in day length as well as in temperature in order to bring the animals as soon as possible into the non-reproductive phase. The day length decreased from 16 hours to 8 hours between May 17 and July 25 and remained constant during the rest of the experiment. The temperature decreased from 20° to 12° C between May 17 and July 1. The latter temperature was then maintained until August 25 in order to be able to carry out salinity tests (this was very difficult at temperatures lower than 12°). On the latter date the temperature was gradually lowered to 4° and this temperature was reached on October 12. It remained constant until October 26 when it was gradually raised to 20°, reaching that temperature on December 19. In Part I, Chapter VI, it was shown that animals in phase 1a do attain maturity under 8 hours day length and 20° C when they have previously been exposed to a period of short day length and low temperature.

a. *Controls.* It can be seen from fig. 18 that in July and August the fish showed a prevailing salt water preference, though not consistently in all sub-tests. This was in agreement with the fact that they had stopped nestbuilding and were losing their nuptial coloration. It can be assumed that the fish would have shown a consistent salt water preference during the following weeks. The number of fish that was used was ten. At the end of November (temperature about 10°) the first two animals built nests. When the remaining fish were tested they showed a consistent fresh water preference. During December all animals built a nest. The temperature was 20° at this time.

b. *Experimentals.* Fig. 18 shows that in the first half of June the fish still preferred fresh water, though not consistently in all sub-tests. In July and August they gradually changed their preference to one for salt water, though again not consistently in all sub-tests. However, it may be assumed that in the following weeks the fish would have shown a consistent salt water preference. In the first week of December the Pt value indicated no preference, but in the last week of that month and in the first week of January the salinity preference had definitely changed to a consistent fresh water preference.

The number of castrated fish that was used in this experiment was 15. In the course of July three of them died. In these fish there was no trace of regenerated gonadal tissue when examined after death. On August 18 one of the remaining fish built a nest. Autopsy revealed a tiny piece of testis tissue and the kidneys were swollen. This fish had been tested in the experiment until then. At the same time two more animals showed a greenish shine (secondary sex character) and were discarded. The remaining 9 fish were used until the end of the experiment and did not show any testis regeneration or swelling of the kidneys when examined at autopsy. Their colour had been brownish all through the experiment (this is the colour of animals in the non-reproductive phase).

The conclusion that can be drawn from this experiment is that a change in salinity preference from salt to fresh water can be induced in gonadectomized fish preferring salt water. There is a difference between the control and experimental groups with respect to the time of the change in preference from salt to fresh water, for the controls start to prefer fresh water earlier than the castrated fish. This point will be discussed on p. 263. The change from fresh to salt water took place somewhat earlier in the controls than in the castrates, though the number of data is too small to allow a definite conclusion.

Experiment 34. In this case the castrated animals were exposed to a constant external condition of 16 hours day length and 20° C. It is known (Part I, Chapter X) that under this condition alternative reproductive and non-reproductive periods occur and it would be interesting to know whether or not there would also be alternative periods of fresh and salt water preference in gonadectomized fish kept under these conditions. Unfortunately, there was no space to keep controls under the same condition, but the effect of such treatment in normal fish has already been shown in experiment 26, fig. 16. The animals described there will be used as controls in the present experiment.

a. *Controls.* Animals kept under 16 hours day length and 20° C have a breeding period of about 90 days (see experiment 30, Part I). In the present animals breeding started around April 15, for at that time the fish built their first nests. This means that the end of their reproductive phase under these conditions would have been around July 15. The salinity preference of animals kept under the above condition during their non-reproductive period has been given in experiment 26, fig. 16. Thus, these data can be used in the case of our present animals, beginning on July 15. They show that the change in preference from salt to fresh water would have taken place in the controls around the beginning of October (see fig. 18).

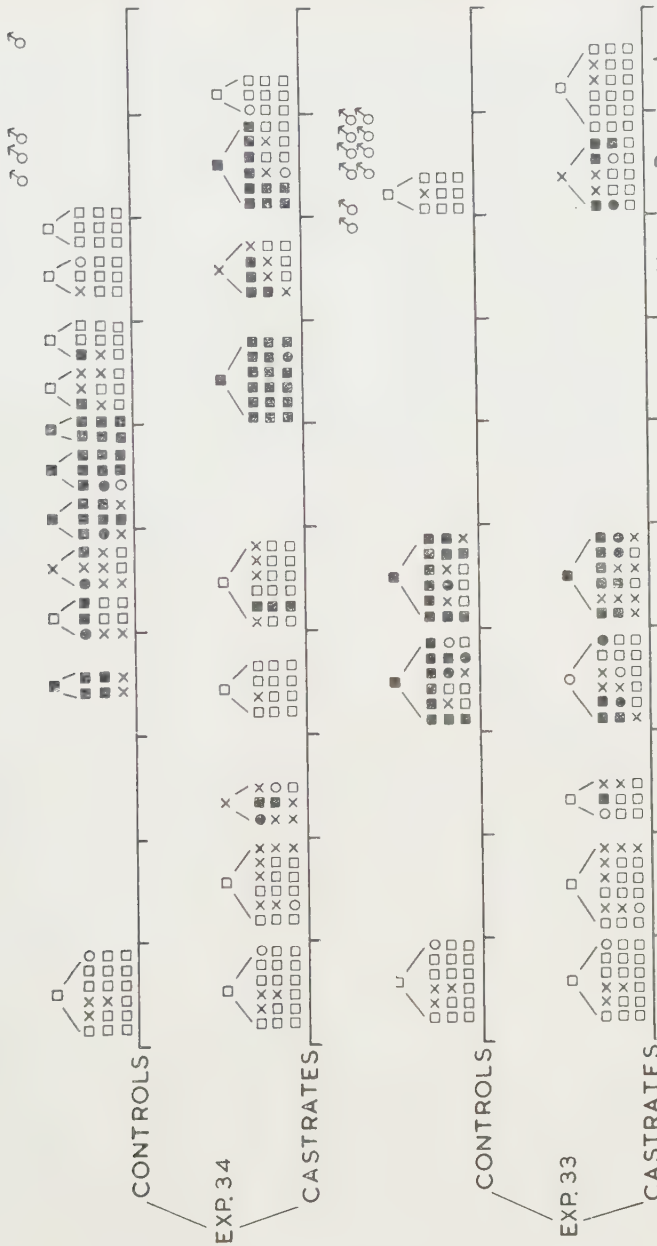

Fig. 18. Cycle of salinity preference in gonadectomized and normal fish maintained under different external conditions. Exp. 33: decreasing day length and temperature, followed by an increase in temperature; exp. 34: 16 hours day length and 20° C. ♂♂: dates of maturity of controls. For further explanation see fig. 12.

b. *Experimentals.* It can be seen in fig. 18 that the Pt value in June indicated no preference, which means that at that time there was a slight tendency to change the initial fresh water preference. However,

in July the animals again preferred fresh water. In August there was again a slight tendency towards a change in the preference, while the definite change from fresh to salt water had taken place in October and presumably earlier. In November and December there were signs which indicated that a gradual change in preference from salt to fresh water was taking place. At the beginning of January the animals showed a consistent fresh water preference.

Autopsy at the end of the experiment showed that in none of the five animals used was there any trace of testis regeneration or of a swelling of the kidneys. The colour of the fish had remained brownish.

The conclusion from this experiment is similar to that of the previous one, namely that a fresh water preference can be induced in gonadectomized animals initially preferring salt water. Though the exact times of the change in preference from salt to fresh water is not known (the dates given here for the controls are those obtained in animals coming from a different group) there is evidence that this change would have taken place earlier in the controls than in the castrated fish, for the difference between the two groups is about two months and it is very unlikely that the time of the change in the present animals would have deviated so much from the average time. A similar difference between the onset of the change in preference from salt to fresh water in castrated and control fish was also demonstrated in the previous experiment.

The change from fresh to salt water preference seemed to have taken place earlier in the controls than in the gonadectomized fish, though, for the same reasons as given above, the exact times are not known. The differences between the times of preference change in castrated and normal fish will be discussed on p. 263 and 264.

There is also evidence that a temporary change in preference from fresh to salt water took place shortly after the operation. This effect will be discussed on p. 265.

It can also be concluded from the two experiments that the same external conditions that are able to induce gonadal maturation are also able to induce in gonadectomized fish a change in salinity preference from salt to fresh water, while those conditions inducing the termination of breeding also induce in castrated fish a change in preference from fresh to salt water.

3. CONCLUSIONS

The main conclusions that can be drawn from the results of the experiments described in this chapter are the following: in animals initially preferring fresh water gonadectomy does not result in a change

in preference from fresh to salt water; it is possible to induce a fresh water preference in gonadectomized fish initially preferring salt water. These results are not expected on the basis of the hypothesis that migration is caused by changes in the gonadal cycle. On the contrary, the experiments have definitely shown that the presence of the gonads is not essential for the induction of migration-disposition.

This fact does not mean, however, that the gonads may not have a modifying effect on the timing of the onset of migration. Evidence for such a modifying influence can be obtained from the above experiments. To this end the times of the changes in salinity preference in gonadectomized and normal fish must be compared (see figs. 17 and 18).

First the onset of the change in preference from salt to fresh water will be considered. This can be done by comparing the results obtained in experiments 33, 34, and 29. It will be noticed that in experiment 33 the normal fish started to prefer fresh water earlier than the gonadectomized fish and the same evidence can be obtained from experiment 34. Though experiment 29 could not be continued long enough it is clear that in this case the change in preference from salt to fresh water also would have taken place earlier in the normal than in the gonadectomized animals.

This effect can be explained in the following way. It is known that gonadal hormones can stimulate the production of gonadotrophic and thyrotrophic hormones by the pituitary (see Burrows, 1949). In some cases it has been shown that small quantities of gonadal hormones had a stimulating effect on the secretion of both pituitary hormones, whereas in other cases there is evidence that large doses had an inhibitory effect. In fishes Fontaine, Lachiver, Leloup and Olivereau (1948) had indications that maturation of the gonads had an inhibiting effect on the activity of the thyroid gland (probably by intermediation of the pituitary).

Assuming for a moment that migration is caused by a hormone we shall give it the name of S-hormone, because the changes in the amount of this hormone are thought to induce changes in the Salinity preference. It must be emphasized here that it is not thought that the S-hormone is a new hormone, but that it is one of the hormones secreted in increased (or decreased) quantities at the time of migration and that its secretion can be influenced by other hormones. From personal experiments described in this chapter it is known that the S-hormone is not a gonadal hormone. It is supposed that its secretion can be influenced by gonadal hormones (either by intermediation of the pituitary or not).

In the case of the above experiments (33, 34, and 29) it is supposed that the amount of hormone produced by the gonads during their

early development was an extra stimulus for the secretion of the S-hormone. This may have been the reason that the normal fish started to prefer fresh water earlier than the gonadectomized animals, the latter having missed this extra stimulus. Later the endocrine system of the gonadectomized animals was able to produce a sufficient amount of S-hormone to induce a fresh water preference without the help of gonadal hormones. In this way it is thought that gonadal hormones may have a modifying effect on the onset of the change in salinity preference, though they are not essential for this change, since it can also be achieved in the absence of gonadal hormones.

Now the onset of the change in preference from fresh to salt water must be discussed. To this end the results obtained in experiments 29, 30, and 31 (see fig. 17) and 34 (see fig. 18) must be compared. These experiments were carried out under external conditions which are known to have a stimulating effect on the secretion of trophic hormones by the pituitary, namely a long or increasing day length and a high or increasing temperature. Experiments 32 and 33 will be left out for a moment, because they were done under decreasing day lengths and temperatures.

In all experiments it can be seen that the change in preference from fresh to salt water takes place earlier in normal than in gonadectomized animals. The effect can be explained by assuming that the presence of a high amount of gonadal hormones in the blood of normal mature fish inhibits the production of the S-hormone which is responsible for the induction and maintenance of the fresh water preference. This inhibiting influence causes a change in preference from fresh to salt water. A tendency towards such a change already takes place in animals shortly after maturation, for it has been shown in experiments 24 and 25 (fig. 15) that mature animals no longer have a consistent fresh water preference. In nature this slight change in preference will not cause seaward migration, because it is weak, and because the external conditions are not favourable for induction of migration-drive (spring or summer conditions).

In the above cases of gonadectomized fish the endocrine system is not affected by a high concentration of gonadal hormones and, therefore, continues to secrete a sufficient amount of S-hormone to maintain the fresh water preference. The continuation is enhanced by the fact that in these experiments the external conditions were still favourable for the secretion of S-hormone. These facts taken together may explain the phenomenon that in the above cases the normal fish started to prefer salt water earlier than the gonadectomized animals. The fresh water preference in the gonadectomized fish is ended by a co-operation between the external conditions and an intrinsic

factor in the same way as the breeding season in normal animals is ended (Part I, Chapter IX, and Part II, Chapter V).

If the above concept of the inhibiting effect of a large amount of gonadal hormones on the secretion of S-hormone is true, why then does the fresh water preference not return as soon as the gonads are regressing? This must be explained by the fact that at that time the endocrine system involved is at the end of its active period (caused by co-operation between external and intrinsic factors) and is no longer able to secrete a sufficient amount of S-hormone to restore the fresh water preference.

In experiment 32 and 33 the fish had been exposed to a decrease in day length as well as in temperature. In 32 the gonadectomized fish started to prefer salt water earlier than the normal fish (thus opposite to the effect in the experiments made under increasing day length and temperature). In experiment 33 the result seems to indicate that the change in salinity preference took place somewhat later in the castrated than in the normal fish. Since the results obtained under these conditions are not in agreement with each other, more data are needed before an explanation can be given of the differences in the onset of the changes in salinity preference found in the above experiments.

The last phenomenon that has to be explained is that in some cases a temporary salt water preference was induced in animals preferring fresh water as a consequence of gonadectomy (experiments 30, 31, and 34). The fact that this effect was shown in animals in which the gonads were developing before the operation and that it did not show in fish with gonads that had been mature for a long time before the operation (experiment 32) or that were about to regress at that time (experiment 29) led to the following explanation.

As was already mentioned the hormones of the developing gonads may have a stimulating effect on the secretion of S-hormone responsible for the induction of a fresh water preference. This stimulating effect must have been present in the above three experiments (30, 31, and 34) at the time of gonadectomy. However, after the operation the stimulating agency suddenly disappeared and the result was a temporary salt water preference or a tendency towards such a preference, because at that time the endocrine system was not yet able to respond to the external conditions alone to produce a sufficient amount of S-hormone. However, after a short time the organism was able, without the help of gonadal hormones, to produce enough S-hormone to ensure a fresh water preference.

4. DISCUSSION

The experiments described in this chapter have conclusively shown that changes in salinity preference, i.e. the induction of migration-

disposition necessary for migration, are not caused by changes in the gonadal cycle. However, there is evidence that gonadal hormones may have a modifying influence on the timing of the onset of migration, while it is possible that they also influence the intensity of the migration-drive (WAGNER, 1956) and migration movements (HOAR *et al.* 1952, 1955).

On the grounds of observations on migrating eels and smolts (migratory stage of young salmon) FONTAINE (1954) concluded that the gonads do not play a primary rôle in the causation of migration. He noticed that adult female eels migrate to the sea when the gonads have just started to develop, while the female smolt migrates with quiescent glands. Moreover, male smolts migrating to the sea have been found with gonads in all phases of development and regression.

In birds castration experiments have also been carried out in order to study its effect on migration (ROWAN, 1932; PUTZIG, 1937, 1938, and 1939; HANN, 1939; DROST, 1941). The results of these experiments generally indicated that in most cases the birds were able to migrate in the right direction after gonadectomy. The direction depended on the time of the year in which the animals were castrated and released. Thus, it can be concluded that in birds gonads also do not seem to play an essential rôle in the causation of migration. However, the number of data on which this conclusion is based is rather small and more experiments are needed before a definite conclusion on this subject in birds can be made.

VII. THE INFLUENCE OF GONADOTROPHIC HORMONES ON SALINITY PREFERENCE

It has been shown that in the Stickleback gonadal hormones do not play an essential rôle in the causation of migration, though they may have a modifying effect on the timing of its onset. There are indications that another hormone, either a pituitary hormone, or one controlled by the pituitary, may be involved in the causation of migration (S-hormone). Now the next step was to find out what known hormone might be identical with the S-hormone. Since there is a close correlation between migration and the gonadal cycle (Chapter V) which is regulated by gonadotrophins secreted by the pituitary, it was decided to test the influence of the latter hormones on salinity preference. These hormones might also play a rôle in gonadectomized animals, because it is known that after this operation gonadotrophic hormones are still secreted and often in even larger doses than before the operation (BURROWS, 1949; BUDDENBROCK, 1950).

The hormones used were Pregnyl, a preparation from pregnancy

urine and Gestyl, a preparation from the serum of pregnant mares. The experiments carried out with these hormones have already been described in detail in Part I, Chapter XI.

Experiment 35. This experiment is the same as experiment 39, Part I, fig. 8. The animals were under-yearling fish that had been reared in the aquarium-house under almost natural conditions. When they were tested around the middle of January they showed a consistent salt water preference (see fig. 19, controls). The administration of hormone (Pregnyl) started on January 9 and lasted until February 2 (for doses, see fig. 8). On February 11 the fish were treated for the second time with the same hormone until February 18 (840 i.u. per 4 hours per day).

a. *Controls*. It can be seen from fig. 19 that in the last week of January the animals still preferred salt water, though less consistently than at the beginning of that month. In February and March the fish showed a fresh water preference. They matured before the middle of March (mean date of maturity of the four fish, March 1).

b. *Experimentals*. Fig. 19 shows that these animals preferred fresh water already at the end of January. This indicates that the change in preference from salt to fresh water took place somewhat earlier in the hormone-treated than in the control fish. The experimental animals matured before the middle of February (mean date of maturity, February 3). This is about one month earlier than the controls.

It was surprising to notice that in the hormone-treated fish the fresh water preference already showed a tendency to change to a salt water preference at the time that the animals attained maturity, unlike the controls at the corresponding time. It is suggested that the administration of gonadotrophin induced an increased production of gonadal hormones, which inhibited the secretion of a sufficient amount of S-hormone to maintain the fresh water preference. Usually this tendency towards a salt water preference occurs after the attainment of maturity (see fig. 15).

The result of this experiment indicates that in case maturation is induced precociously by means of gonadotrophins, the salinity preference is also prematurely induced to change from salt to fresh water. This does not mean, of course, that the gonadotrophic hormone has been the direct cause of this change in salinity preference, for it may have stimulated the secretion of S-hormone, which in its turn would have changed the preference. The stimulation of the S-hormone may have been direct or via the intermediation of gonadal hormones.

The experiment only indicates that gonadotrophic hormone influenced the time of the change in salinity preference, while by doing so it did not act differently from a condition such as a long day length

combined with a high temperature. Neither stimulus induces an immediate change in preference, but manifests itself only after a certain period of time. This action is unlike that of the S-hormone, as will be shown in the next chapter.

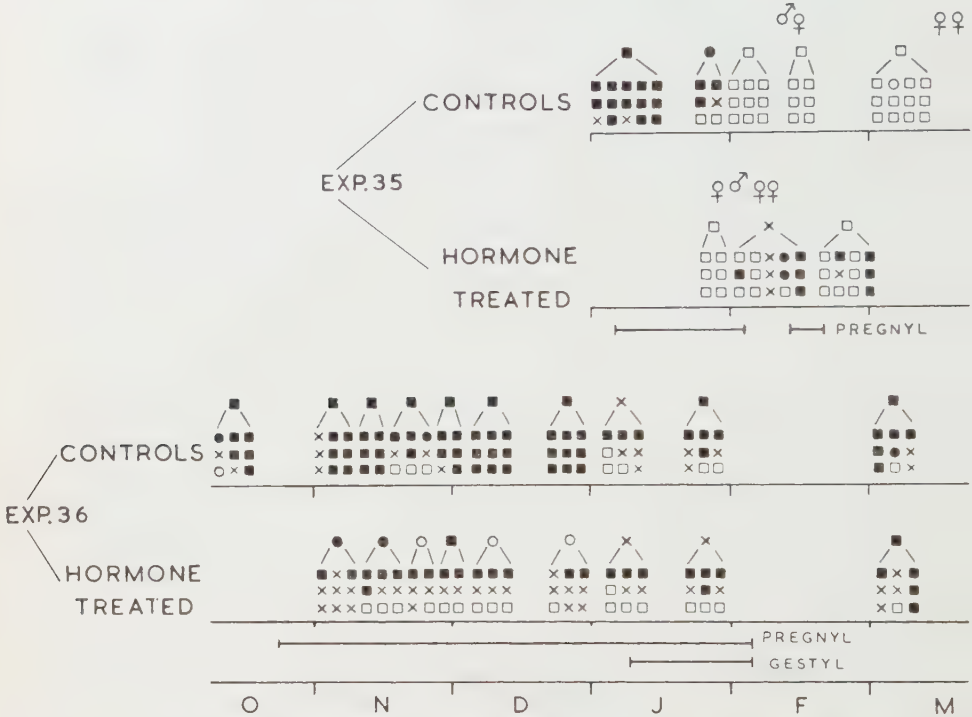

Fig. 19. Salinity preference of fish in different phases of gonadal development treated with mammalian gonadotrophins. Exp. 35: fish in phase 2, almost mature. Exp. 36: animals kept in phase 1 by exposing them to 8 hours day length and 20° C. The duration of the hormone treatment is indicated by horizontal lines. ♂ and ♀: dates of maturity. For further explanation see fig. 12.

Experiment 36. This experiment is the same as experiment 40, Part I. The animals used had been kept for a long period of time under 8 hours day length and 20° C without attaining maturity. In Part I, Chapter VI, it was shown that this external condition inhibits maturation in animals exposed to it in phase 1 of gonadal development. In Part II, Chapter V, it has been shown that when animals are exposed to this condition at a very early stage of gonadal development (phase 0 or the beginning of 1a) they maintain their initial salt water preference, even when kept for over one year under the same condition. Now it was decided to administer gonadotrophins to such animals in

order to find out whether these hormones could induce maturation and/or a change in preference from salt to fresh water.

a. *Controls*. Fig. 19 shows that these fish maintain their salt water preference all through the experiment, though in some sub-tests they preferred fresh water. Only at the beginning of January the Pt value indicated no preference. None of the fish matured in the course of the experiment.

b. *Experimentals*. The first thing that can be noticed is that at no time in the course of the experiment did the animals show a consistent fresh water preference, though the Pt values of three test series were below the 0.05 level for fresh. However, even in these cases the preference was far from consistent in the sub-tests. On the whole it seems as if the hormone-treated fish showed a greater tendency towards a change in preference from salt to fresh water than the controls. This might indicate that the administration of gonadotrophins had resulted in a slight change in the salinity preference. It cannot be concluded whether this effect of the gonadotrophic hormones was direct, or whether it was an indirect effect of increasing the secretion of gonadal hormones, which in their turn might have stimulated the output of S-hormone. None of the hormone-treated fish matured.

The result of this experiment shows that under 8 hours day length and 20° C the administration of gonadotrophic hormones neither resulted in a definite change in salinity preference nor in the induction of maturation. For a possible explanation of this negative effect see p. 205. The experiment must be repeated with hypophysectomized and gonadectomized animals before a conclusion on the possible rôle of gonadotrophic hormones in the causation of migration can be made.

However, in neither of the two experiments were there any indications that these hormones had an immediate effect on salinity preference, for a considerable time elapsed (3–4 weeks) between the first administration of the hormones and the first signs of a tendency towards a change in salinity preference. Therefore, it is more likely that gonadotrophins act via the intermediation of the S-hormone and are not primarily involved in the causation of migration, though they may have a modifying effect via stimulation of the gonads.

VIII. THE INFLUENCE OF THYROXINE AND THIOUREA ON SALINITY PREFERENCE IN NORMAL AND GONADECTOMIZED ANIMALS

Now that it has been demonstrated that the gonads do not play an essential rôle in the causation of migration-disposition, while there is

some evidence that gonadotrophic hormones are also not essential, the effect of other hormones on salinity preference had to be investigated. It has been mentioned in Chapter II that in fishes migration is often correlated with changes in their osmotic regulation. Besides, it was observed that migration is also very often correlated with changes in the activity of the thyroid gland, while in the case of the Stickleback Koch and Heuts (1942) were able to show that feeding of thyroid containing substance resulted in a decreased ability for osmotic regulation. It was therefore decided to study the effect of thyroxine and thiourea (thyroid inhibitor) on salinity preference in the Stickleback.

Before the experiments are described a short description will be given of the way in which thyroxine and thiourea were administered. A synthetic preparation of thyroxine made by Hoffmann-La Roche (Basel) was used. Thyroxine as well as thiourea was dissolved in tap water that was held in 10 l battery jars. In each jar a group of 5 fishes was kept. The water was renewed each day in both the experimental and control groups. The animals were fed once a day with live Tubifex and the water was well aerated.

I. THE INFLUENCE IN NORMAL ANIMALS

In the following two experiments the fishes were kept in the aquarium-house where conditions were almost similar to those in nature (day length about 10 hours; temperature 9° C ± 0.5). The fish used were under-yearling animals hatched in April and May, 1955. They had been maintained in the aquarium-house under almost natural conditions (see fig. 1). When the experiment started on October 15 they measured on the average 5.0 cm. At that time they showed a prevailing salt water preference, as can be seen in fig. 20. With this group of fish two different experiments were started, namely 37 and 38, each of which consisted of two parts, A and B.

Experiment 37 A. On October 17 one lot of five animals was placed in a solution of thyroxine 1 : 1.000.000. On October 21 this concentration was doubled. The results of the salinity tests are given in fig. 20. The fish were tested for the first time on the fourth day of the treatment. On that day they preferred salt water in one sub-test and had no preference on the other two. But on the fifth day the preference had changed to a consistent fresh water preference and this remained during the next three days.

The result shows that thyroxine has an almost immediate effect and probably also a direct effect on salinity preference by inducing

a fresh water preference, while the controls continued to prefer salt water.

Experiment 37B. The animals from 37A were placed for one day in tap water without any dissolved preparation after the thyroxine treatment had stopped on October 24. The next day they were placed in a solution of 0.036 % thiourea, while the concentration was doubled the following day. They were tested for the first time on the third day of the treatment and showed a fresh water preference in two sub-tests and had no preference in the third. However, on the fourth day of the treatment the preference had changed to a salt water preference and this remained during the following four days.

This result might indicate that thiourea induced a salt water preference in animals preferring fresh water. However, the experiment is not conclusive on this point, because it is possible that the salt water preference was restored after the thyroxine treatment had stopped and thus might have been achieved without the influence of thiourea. However, in experiment 39 it will be demonstrated that thiourea is indeed able to induce a salt water preference and, therefore, it is very likely that it will also have done so in the above case.

Fig. 20 shows that in the course of experiment 37 the controls

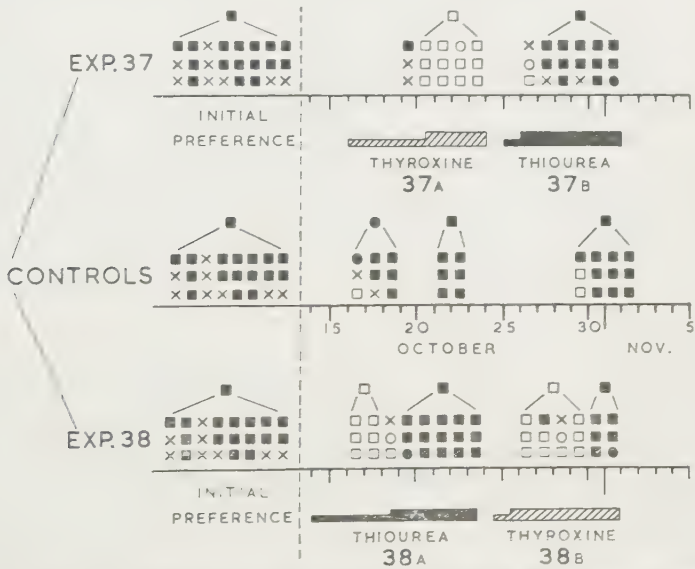

Fig. 20. Salinity preference of fish initially preferring salt water, treated with thyroxine and thiourea. Black columns: duration of thiourea treatment; hatched columns: duration of thyroxine treatment. Height of columns indicates the amount of substance used. For further explanation see fig. 12.

maintained their salt water preference, though in some sub-tests they had either no preference or even a fresh water preference. However, the Pt values were below the 0.01 level for salt water.

Experiment 38A. In this experiment the animals were first placed in a thiourea solution. The experiment started on October 15. During four days the concentration of the solution was 0.036%, while it was doubled on the fifth day. Fig. 20 shows the surprising result that on the third and fourth day of the treatment the animals had changed their initial salt water preference to a fresh water preference. The preference started to change again on the fifth day and during the following five days the fish showed a consistent salt water preference.

This temporary change in preference at first seemed difficult to explain. However, it is known that thiourea stimulates the epithelial cells of the thyroid gland, though it prevents the synthesis of thyroid hormone (CHARIPPER and GORDON, 1947; LEVER *et al.*, 1949; see also Mem. Soc. Endocrinol. No. 1, 1953). It is known that the reduction in the amount of thyroid hormone in the blood is counteracted by an increased secretion of thyrotrophic hormone (see BUDDENBROCK, 1950). The effect of administration of thiourea is twofold. In the first place it releases the stored colloid in the thyroid gland and in the second place it causes an increase in the secretion of thyrotrophin.

Now it is suggested that in the above case the temporary change in salinity preference from salt to fresh water under the influence of thiourea is caused by the release of the stored colloid. After this had been consumed the initial salt water preference returned. HOAR *et al.* (1952) observed a similar effect when they treated young salmon with thiourea. This substance caused an increase in the rate of swimming during the first days after the treatment had started, while later it reduced the activity until it was below normal. This was in agreement with the fact that they observed an increase in general activity after administration of thyroxine.

The salt water preference in the above experiment that returned on the sixth day of the treatment can be explained in the way described in the previous paragraph, though it is also possible that it might have been induced by complete inhibition of the formation of thyroid hormone by the action of thiourea. That the latter way of induction might have taken place is supported by the fact that the salt water preference was more pronounced during the thiourea treatment than before the experiment started. Besides, it was more consistent than in the controls.

Experiment 38B. The thiourea treatment was stopped on October 24

and the next day the fish were treated with a 1 : 1.000.000 concentration of thyroxine. This was doubled the following day. Fig. 20 shows that already on the third day of the thyroxine treatment the fish had changed their preference and preferred fresh water. This preference lasted during the next three days, but on the seventh day the animals suddenly started to prefer salt water and the preference was the same on the next day when the experiment was terminated.

The fresh water preference on the third and following days is the direct effect of the thyroxine treatment and agrees with what was found in experiment 37 A. However, the return to a salt water preference on the seventh day is difficult to explain. It is possible that the previous treatment with thiourea affected the thyroid gland unfavourably in that it was not able to respond very long to the thyroxine treatment (a kind of after-effect, see also p. 274).

The main conclusion from the experiments is that thyroxine is able to induce a fresh water preference in animals preferring salt water. The experiments are not conclusive as to the rôle of thiourea, because the fish preferred salt water initially. Therefore, in the next experiment the effect of thiourea in animals initially preferring fresh water will be described. This experiment will also give some information on the after-effect of the thiourea treatment.

Experiment 39. The animals used in this experiment hatched in April and May 1955 and were reared in the aquarium-house under almost natural conditions (see fig. 1). On February 10 of the next year the fish were transferred to 16 hours day length and 20° C in order to induce them to prefer fresh water (Chapter V). When they were tested on February 17 they showed a preference for fresh water (see fig. 21).

One lot of five animals was treated with 0.072 % thiourea and a second lot was kept as a control. The external conditions remained the same.

It can be seen in fig. 21 that during the first week the controls maintained their fresh water preference, unlike the thiourea-treated fish. When the latter were tested on the third day of treatment they had already changed their preference to a consistent salt water preference and this remained during the next two days. The result shows conclusively that thiourea is able to induce a salt water preference in animals initially preferring fresh water.

The thiourea treatment ended after five days, i.e. on February 22. The animals were replaced in the aquarium together with the controls; both groups were marked. After five and ten days respectively the former thiourea-treated fish still preferred salt water (see fig. 21) and none of them matured during this time.

However, during the same time three out of the five control animals matured and the remaining two attained maturity a little later. This was a rather unfortunate circumstance, since it meant that the fresh water preference of these fish would become less prominent, as it always does in mature fish (see fig. 15). That this did indeed happen in the present experiment is shown in fig. 21, where it can be seen that on February 27 the animals did not show any preference, while they had a prevailing salt water preference on March 3.

This tendency towards a salt water preference in the controls made comparison with the former thiourea fish rather difficult. However, the fact that the latter animals maintained their salt water preference and did not mature during the first two weeks after treatment indicates that administration of thiourea has a long after-effect on salinity preference and on maturation. This might have been the reason that in experiment 38B administration of thyroxine following treatment with thiourea resulted in only a temporary change in salinity preference (see fig. 20).

In the above experiment it was evident that maturation of the gonads had been delayed by the treatment with thiourea, for the first fish in this group did not mature until March 15, whereas the last fish in the control group attained maturity on March 14. Unfortunately, the remaining four experimental fish died one night, most probably because of a kind of food poisoning. A retardation of development of secondary sex characters and thus of gonadal development by means of thiourea has also been reported by Goldschmidt *et al.* (1944); Nigrelli *et al.* (1946), and Hopper (1950, 1952). This effect is more or less conceivable since it is known that thyroid hormone often has a stimulating effect on gonadal development (Burrows, 1949; Buddenbrock, 1950).

The conclusion from this experiment is that thiourea is able to induce a salt water preference in animals preferring fresh water. There is evidence that maturation in animals treated with thiourea is delayed as a consequence of this treatment and that it has a considerable after-effect on salinity preference. This experiment confirms the results of previous experiments which indicated that thiourea might be able to induce a salt water preference.

Experiments 37 and 38 were carried out under natural decreasing day lengths (about 10 hours at the beginning of the experiment) and a temperature of about 9° C. Experiment 39 was carried out under 16 hours day length and 20° C. In the case of treatment with thiourea these differences in external conditions during the experiments did not have an essential influence on the effect of the treatment. The difference in the duration of the latency periods, i.e. the time needed to respond, will be dealt with on p. 278.

The next two experiments were done in order to study the effect of a thyroxine treatment under external conditions different from that in experiments 37 and 38 (day length about 10 hours; temperature about 9° C).

Experiment 40. The animals used were under-yearling fish that had hatched in the laboratory in April and May, 1955 and had been reared in the aquarium-house under almost natural conditions (see fig. 1). On July 11 they were transferred to 8 hours day length and 20° C. At the time of the transfer the animals had been in phase 1 of gonadal development, for they did not mature under the new condition (see table 5).

When the fish were tested around November 12 they showed a prevailing salt water preference, as can be seen in fig. 21. On November 13 some of the animals were placed in a solution of 1:1.000.000 thyroxine. This lasted for 6 days, while the concentration was doubled on the seventh and eigth day.

a. *Controls.* It can be seen in fig. 21 that the controls maintained their salt water preference during the above period, though it was not consistent in all sub-tests.

b. *Experimentals.* Fig. 21 shows that when the thyroxine treated fish were tested on the third day of the treatment they still preferred salt water, but that on the fourth day there was a tendency to change the salinity preference. On the fifth day the preference had definitely changed and the fish now preferred fresh water. This lasted for the next three days.

This experiment confirms the results obtained in experiments 37 and 38 which also showed that thyroxine given to animals preferring salt water resulted in the induction of a fresh water preference. The external conditions under which the experiments were carried out evidently did not influence the result of the treatment.

Experiment 41. Under-yearling fish were used that had hatched in the laboratory in April and May, 1955 and had been reared under almost natural conditions. On July 11 they were transferred to 16 hours day length and 20° C. When the fish were tested on November 12 they showed a weak salt water preference. This meant that they were not far from attainment of maturity. Though the weak salt water preference was not very suited to demonstrate the effect of thyroxine, it was nevertheless decided to carry through the experiment.

On November 13 some of the fish were treated with thyroxine 1:500.000, while the others were kept as controls.

a. *Controls.* It can be seen in fig. 21 that during the experiment the

animals changed their preference to a consistent fresh water preference. The fish did not mature in the course of the experiment, though they all attained maturity within the next two months.

b. *Experimentals*. Fig. 21 shows that on the fifth day of the treatment

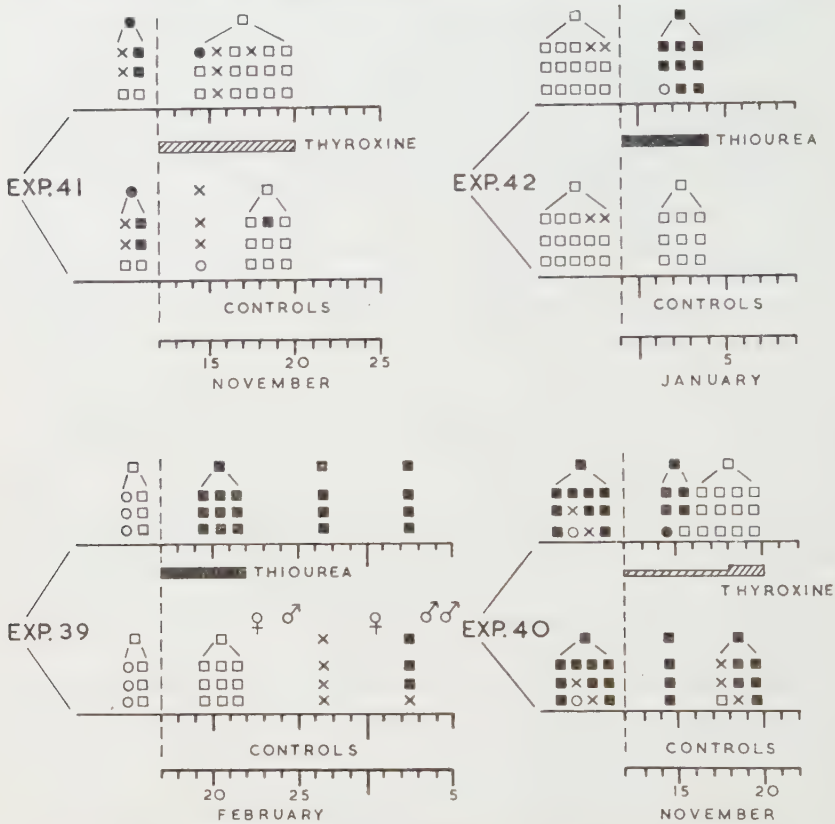

Fig. 21. Salinity preference of fish kept under different external conditions and treated with either thyroxine of thiourea. Exp. 39; 40, and 41: normal fish; exp. 42: gonadectomized fish. For further explanation see fig. 12.

the fish had a consistent fresh water preference and this remained for the next three days when the experiment was terminated.

The result of this experiment is not fully conclusive, since the difference between the treated fish and the controls was small. However, it is evident that thyroxine has the same effect in animals about to prefer fresh water and to attain maturity, as in fish preferring salt water and far from the attainment of maturity (experiments 37, 38, and 40). The different external conditions used in the latter experi-

ments and the present one do not seem to have affected the result of the thyroxine treatment. With respect to treatment with thiourea a similar conclusion has been drawn.

2. THE INFLUENCE OF THIOUREA ON GONADECTOMIZED ANIMALS

In the above experiments normal animals were used and it is possible that the presence of the gonads could have played a rôle in the induction of changes in salinity preference under the influence of thyroxine and thiourea. Therefore, it was decided to repeat the experiments with gonadectomized fish in order to exclude a possible intervention of the gonads. Unfortunately, there was no opportunity to study the effect of thyroxine in gonadectomized animals that prefer salt water, but this could be done with respect to the effect of thiourea in gonadectomized fish preferring fresh water.

Experiment 42. The animals used were the same as those used in experiment 33 (see fig. 18) in which a fresh water preference was induced by 8 hours day length and 20° C following exposure to a period of short day length and low temperature. When the animals were tested at the end of December and the beginning of January they showed a consistent fresh water preference (see fig. 21).

On December 31 some of these fish (5) were treated with 0.072 % thiourea and this lasted until January 4. The rest of the fish were kept as controls. All fish remained under 8 hours day length and 20° C.

a. *Controls.* In fig. 21 it can be seen that the controls maintained their fresh water preference all during the experiment.

b. *Experimentals.* When the fish were tested for the first time on the third day of the treatment they had already changed their preference to a prevailing salt water preference and on the fourth and fifth day they showed a consistent salt water preference (see fig. 21).

The result of this experiment shows that in gonadectomized animals preferring fresh water treatment with thiourea is able to induce a salt water preference. This means that there is no difference between the effect of this substance on normal and gonadectomized fish. Thus, the gonads are not essential for the induction of a salt water preference by means of thiourea.

Though the effect of thyroxine on gonadectomized fish that prefer salt water could not be studied, it can be safely assumed that this treatment would have resulted in the induction of a fresh water preference.

3. THE LATENCY PERIODS OF THE EFFECT OF THYROXINE AND THIOUREA UNDER DIFFERENT CONDITIONS

The previous experiments have shown that the effect of thyroxine as well as thiourea on salinity preference manifests itself a few days after the beginning of the treatment. In table 10 the latency as shown in the experiments described in this chapter are given, together with details on the condition of the fish at the beginning of the treatment, the external condition during the treatment etc. The results of the thyroxine treatment in animals about to prefer fresh water (41) and those of the thiourea treatment in fish already showing a salt water preference (38A) are not given.

TABLE 10

Latency of the onset of changes in salinity preference after administration of thyroxine or thiourea.

No of experiment	*External conditions*		*Condition of animals*	*Previous treatment*	*Latency thiourea*	*Latency thyroxine*
	day length	*temp. in °C*				
37 A	± 10 h.	9 ± 0.5	immature	none	—	5 days
37 B	± 10 h.	9 ± 0.5	immature	thyroxine	4 days	—
38 B	± 10 h.	9 ± 0.5	immature	thiourea	—	⩽ 3 days
39	16 h.	20	almost mature	none	⩽ 3 days	—
40	8 h.	20	immature	none	—	5 days
42	8 h.	20	gonadectomized	none	4 days	—

Table 10 shows that in normal and gonadectomized animals the latency of the effect of thiourea is about 3 to 4 days. It seems that the different external conditions did not affect the duration of the latency period very much, though the result of experiment 39 indicate that this period is shortest under conditions known to have the most stimulating effect on the activity of the pituitary (16 hours day length and 20° C, see Part I, Chapter IV). However, in this case the fish were almost mature, unlike those in experiments 37 and 42. More data must be obtained before a conclusion on the influence of both external and internal conditions on the duration of the latency period of thiourea treatment on salinity preference can be made.

The same holds for the duration of the latency of thyroxine, where the number of data is also too small to draw a conclusion. Table 10 shows that in two cases the latency period lasted 5 days, while it was three days in the third case. It is rather surprising to notice that the shortest duration of the latency period was obtained in fish that had previously been treated with thiourea (38B). It is known that thiourea stimulates the activity of the epithelial cells, though it inhibits the

synthesis of thyroid hormone. It is possible, therefore, that in the above case, as soon as the inhibitory effect of thiourea was abolished, the hyperactivity of the epithelial cells together with thyroxine caused an accelerated formation of thyroid hormone. However, if this were true it is difficult to explain the inability of thyroxine to maintain the induced fresh water preference longer than four days (experiment 38B, see fig. 20).

In the literature (see BUDDENBROCK, 1950) there are data showing that the duration of the latency period of thyroxine administration depends on the mechanism studied (e.g. metabolism; respiration etc.) and on the quantity of the administered thyroxine. Besides, the responses may be different in the different species studied.

Thiourea also needs some time before it can exert its maximum effect. FORTUNE (1953, 1955) found that the speed of reaction to thiourea treatment (increase in height of the epithelial cells) in *Phoxinus laevis* was more rapid at higher than at lower temperatures. OLIVEREAU (1955c), however, criticized the result of FORTUNE's experiment by stating that the histological criterion the latter author used was not appropriate for this study. OLIVEREAU (1954) studying the effect of thio-derivates in different species of Teleosts with the help of radioactive iodine was unable to show any effect of temperature on the speed of inhibition of the secretion of thyroid hormone. Therefore, it is at the moment not yet possible to decide whether the short latency period of thiourea in experiment 38B (see table 10) was due to the high temperature used, or to other factors such as day length and state of gonadal development.

4. THE MECHANISM OF THE HORMONAL INFLUENCE ON SALINITY PREFERENCE

Fish migrating between fresh water and the sea must be able to regulate the osmotic concentration of their body fluids in accordance with the changes in the external medium. Teleost fishes living in fresh water have a body fluids concentration that is hypertonic to that of the external medium, while the concentration of the body fluids in marine fish is hypotonic to that of the external medium. In fresh water fish the hypertonicity is accomplished by acquisition of salts from food and from the surrounding medium by means of permeable membranes and chloride accumulating cells in the gills. The excess of water is removed by excretion of hypotonic urine. In marine fish the hypotonic concentration of the body fluids is accomplished by swallowing water and excreting salt in the feces, urine and by the salt excreting cells in the gills. The salt accumulating cells are identical with the salt excreting

cells. The mechanism of osmotic regulation has been discussed and reviewed by SMITH (1932), KROGH (1939), FONTAINE and KOCH (1950), BLACK (1951), and FONTAINE (1956).

The fact that Sticklebacks show salinity preference gives rise to at least three questions, namely *a.* how do the animals perceive the differences in salt concentration and behave accordingly, *b.* why do they show a salt water preference at one season and a fresh water preference at another, and *c.* in what way can hormones, like the thyroid hormone, affect the salinity preference? We shall deal with these questions separately.

a. *The discrimination between various degrees of salinity*

The fact that Sticklebacks show pertinent salinity preferences means that they must be able to perceive the salt concentration of the medium. How do they perceive the different concentrations? In the first place it is possible that they have a peripheral sense organ, most probably the gustatory organ, which perceives the salt concentration. A second possibility is that the salt concentration of the medium is perceived more centrally, when the concentration and the chemical composition of the blood change due to contact with water of a different salinity. It is known that mammals possess chemical receptors in the central nervous system and in the carotid sinus, and they might exist in fishes as well.

However, with regard to the salinity preference it seems rather unlikely that this type of perception would play a rôle, since in the tests the fish can often be seen reacting immediately (e.g. by swimming away) after making contact with water of different salinity. Such an immediate response is difficult to conceive if the perception is mediated by changes in osmotic concentration of the blood. Therefore, the first possibility seems to be the most likely one.

It has been shown that many fishes are able to distinguish between very small differences in the salt concentration of the external medium (KRINNER, 1934; BULL, 1937).

The fact that the fish show preference for a certain degree of salinity also implies that a particular behaviour pattern must have been activated. Thus, perception of the concentration of the external medium is dealt with by the central nervous system and the behaviour induced is such that the fish are repelled by a certain salt concentration and/or attracted by another concentration.

b. *The alternative cycle of salt and fresh water preference*

Why do the fish prefer salt water at one time and fresh water at another time? The first possibility is that a change may have taken

place in the mechanism regulating their osmotic equilibrium, so that they are no longer adapted to their external medium. If the fish were living in fresh water at the time of the disturbance of the osmotic equilibrium, the concentration of the body fluids would decrease, while if they were living in salt water, the latter concentration would increase. Now it might be possible that a salt concentration of the body fluids below the level maintained in fresh water would affect the central nervous system in such a way that it induces a salt water preference. A concentration of the body fluids higher than the level maintained in sea water would induce a fresh water preference. However, it is also possible that the changes *as such* in the osmotic regulation mechanism and not the changes in concentration or chemical composition of the body fluids would affect the central nervous system.

If one of these possibilities were true the changes in the osmotic regulation system would be the direct cause of the changes in salinity preference and thus of induction of migration-disposition. As will be shown in the next section a disturbance of the osmotic equilibrium occurs in many diadromous fishes at the time of migration, among them the three-spined Stickleback.

It is interesting that in migrating Crustaceans the osmotic equilibrium also becomes disturbed at the time of migration (Koch and Heuts, 1944; Fontaine, 1954). It must be emphasized that in nature disturbance of the osmotic equilibrium need not necessarily lead to the death of the animals, though it may kill some of the fish (see Hoar, 1952.

A second possibility is that at the time of migration a change takes place in the central nervous system under the influence of certain stimuli (though not of a disturbance in the osmotic equilibrium) which induces the above type of behaviour by which the fish show preference for a different salinity concentration than they preferred initially. In this case disturbance of the osmotic equilibrium would not be the primary factor causing changes in salinity preference, but a secondary phenomenon enabling the fish to adapt to the new environment.

c. *Thyroid hormone and the way in which it may affect the salinity preference*

It was mentioned above that changes in salinity preference might be caused either by changes in the osmotic equilibrium, or by other factors than disturbance of the osmotic regulation system. It has been shown in the experiments described in the present chapter that the amount of thyroid hormone present in the blood affects the salinity preference. This effect may be achieved either by influencing the osmotic system and thus, indirectly, the central nervous system, or by

influencing directly the latter system. These two possibilities will be discussed separately.

i. Influence on osmotic regulation

Koch and Heuts (1942) have shown that the administration of thyroid substance to Sticklebacks living in salt water induced a change in their capacity for osmotic regulation, so that chlorides penetrating from the external medium were no longer excreted, but accumulated in the blood and finally caused the death of the animals. Sticklebacks living in fresh water did not suffer any harm from the feeding of thyroid substance. It is evident, therefore, that the thyroid hormone directly affects the osmotic system. Now it might be possible that thyroid hormone influences the salinity preference by causing a disturbance in the osmotic equilibrium, though there is as yet no proof for this assumption.

A further question is in what way thyroid hormone may affect osmotic regulation. Investigations on osmotic regulation of fresh water and marine Teleosts have shown that in the majority of animals the special cells present in the gills (chloride excreting or accumulating cells) play an important rôle. Now it is suggested here that thyroid hormone would affect these cells. In this case there are at least two ways in which these cells may be affected. In the first place it is possible that the amount of hormone present in the blood would determine the manner (excretion or accumulation) in which these cells deal with chlorides. In spring, when there is much thyroid hormone in the blood (see section 5 of the present chapter) the cells would be induced to accumulate chlorides and thus cause a disturbance in the osmotic equilibrium. In autumn the low concentration of this hormone would cause the cells to excrete chlorides and thus also cause a disturbance of the osmotic system. Such a method by which thyroid hormone might influence the osmotic regulation may hold for animals that have a hyperactive thyroid in spring and a hypoactive gland in autumn.

However, in animals that have active thyroid glands both at the time of seaward and of fresh water migration such a mechanism is more difficult to conceive, though it remains possible that differences in the activity of this gland in spring and autumn would cause the above effect. But it can also be suggested that in the case of these fishes a large amount of thyroid hormone has an inhibiting effect on the *active* process of chlorides excretion or accumulation and thus prevents the animals from regulating the osmotic concentration of their body fluids in accordance with the external medium.

No decision can yet be made between the two mechanisms described above, while it remains possible that the osmotic regulation is affected

by other hormones, either singly or in co-operation with the thyroid hormone, or in still another way.

ii. Direct influence on behaviour

In this case the thyroid hormone would act directly upon the central nervous system and induce a certain type of behaviour by which the animals show a preference for either salt or fresh water. At the same time the osmotic equilibrium is affected, but this is a secondary effect in this case.

It is generally thought (see BEACH, 1948) that hormones may affect the responsiveness of the nervous system to external stimulation, while usually an increased amount of hormone intensifies the drive of the behaviour which is affected by the hormone.

With respect to the possible action of thyroid hormone on migratory behaviour it could be suggested that the nervous centres inducing the behaviour are affected by this hormone. In the case of the Stickleback it is possible that a high amount of thyroid hormone would influence the centre responsible for the induction of fresh water migration, while a low amount of this hormone would affect the centre inducing seaward migration.

However, in species of fish having hyperactive glands both at the time of seaward and of fresh water migration, it is more difficult to conceive the way in which a high amount of thyroid hormone would give rise to two different behaviour patterns. Nevertheless, it remains possible that in this case there might still be a quantitative difference between the amount of hormone secreted at the time of seaward and of fresh water migration.

It is also possible that the hormone would affect the general activity of the fish. It then would not influence the responsiveness of nervous centres as described above, but would affect the sensitivity of the perceptual mechanism by which the fish become sensitive to certain external stimuli that may direct their general activity. Also in this way it is possible that thryoid hormone may influence migratory behaviour (see also HOAR *et al.*, 1952 and 1953).

It may be remarked here that the possible induction of migratory behaviour by disturbances in the osmotic equilibrium may be of importance only in diadromous fish. However, the other possibility that thyroid hormone might affect migratory behaviour by a direct influence on the central nervous system can be more universally applied to diadromous as well as to potamodromous and oceanodromous migration, thus of migration taking place in only one medium (terminology of MEYERS, 1949).

Finally it must be emphasized that until now no behavioural

response has been found that depended on the action of only one hormone, while on the other hand one hormone usually exerts more than one effect upon the organism (Beach, 1948). Thus in the case of migration it is possible that besides the thyroid hormone other hormones may act simultaneously on the central nervous system or on other mechanisms that may play a rôle in the causation of migration, while non-hormonal stimuli may be involved as well.

5. CONCLUSIONS AND DISCUSSION

The experiments described in this chapter have conclusively shown that thyroxine and the thyroid inhibitor thiourea have an almost immediate effect on salinity preference, unlike the external conditions and the gonadotrophic hormones used in experiments described in previous chapters. Thyroxine induces a fresh water preference and thiourea a salt water preference. This induction can be achieved in the absence of the gonads.

The results are in full agreement with those obtained by Koch and Heuts (1942, 1943), and Heuts (1943). These investigators were able to show that the osmotic regulation becomes less effective during the reproductive season in *Gasterosteus aculeatus* as well as in *Pygosteus pungitius*. Guyelard (1924), cited by Fontaine and Koch (1950), has also been able to demonstrate that the 3-spined Stickleback showed a reduced euryhalinity in the reproductive season.

Further Koch and Heuts were able to show that in both species of Sticklebacks living in sea water, feeding of thyroid substance caused a reduced capacity for osmotic regulation. The reaction of *Pygosteus* was different from that of *Gasterosteus* in some points, but in the former species there was also a tendency towards a reduced effectiveness of the osmotic system. It must be remarked that *Pygosteus* is not a true migratory species.

Finally, the above mentioned authors were able to show (by using histological criteria) that in spring the thyroid gland of *Gasterosteus* is more active than in winter. Unfortunately nothing is known about the activity of the thyroid in this species at the time of seaward migration.

All results mentioned here are in full agreement with the fact that thyroxine induces a fresh water preference in animals living in salt water. If it could be shown that at the time of seaward migration the thyroid gland is quiescent, or less active than in spring, this fact would be in full agreement with the induction of a salt water preference by thiourea, which inhibits the formation of thyroid hormone.

The observation of Koch and Heuts that the thyroid gland is

active in spring does not show conclusively whether it is the increased amount of thyrotrophin or the increased amount of thyroid hormone which induces the change in osmotic regulation at this time. However, there is evidence that the changes in salinity preference are caused by thyroid hormone and not by thyrotrophin. For it is known that when thyroxine is administered the increased amount of thyroid hormone is counteracted by a decrease in the amount of thyrotrophic hormone in the blood. On the other hand a decrease in the amount of thyroid hormone in the blood (caused by thiourea, for example) is followed by an increased secretion of thyrotrophin (Buddenbrock, 1950). This means that when thyroxine is administered the amount of thyrotrophic hormone in the blood would decrease. Thus if the change in salinity preference were due to thyrotrophic hormone, instead of to thyroid hormone, this would mean that in the experiments the change in salinity preference from salt to fresh water would be due to a low amount of circulating thyrotrophin, while in nature (where the thyroid gland is active at the time of migration to fresh water, and thus much thyrotrophin must be circulating) the same change would be due to a high amount of thyrotrophin in the blood. However, in both cases the amount of thyroid hormone in the blood is high and it is, therefore, much more likely that the change in salinity preference from salt to fresh water is due to a high amount of thyroid hormone in the blood. In case there is little thyroid hormone present, (presumably in autumn, and during a thiourea treatment) a salt water preference is induced. Smith (1956), on the contrary, believes that the thyrotrophic and not the thyroid hormone is involved in salinity tolerance.

The results of all experiments on salinity preference described in previous chapters can easily be explained in terms of an increase or decrease in the amount of thyroid hormone. In the cases where external conditions were able to induce a change in salinity preference (namely in spring) it is assumed that these conditions affected the secretion of thyrotrophic hormone by the pituitary and thus caused an increased secretion of thyroid hormone. This means that the secretion of thyrotrophin depends on stimulation by the same external factors as the secretion of gonadotrophins (see Part I).

When an intrinsic factor in co-operation with external factors is responsible for the change in salinity preference (namely at the end of the breeding season) it is assumed that a kind of exhaustion of the pituitary caused a decreased output of thyrotrophic hormone and thus of thyroid hormone. It is also clear why it is possible that changes in salinity preference can be induced in gonadectomized fish by proper manipulation of external factors or by administration of thiourea and probably of thyroxine. However, the gonads may have a modifying

influence on the timing of the onset of the changes in salinity preference most probably by affecting the amount of thyrotrophin secreted by the pituitary, and thus of the amount of thyroid hormone. For details see Chapter VI (Part II) where in the explanation of the modifying rôle of gonadal hormones on the onset of changes in salinity preference the term S-hormone must be replaced by thyroid hormone.

The present chapter will be ended by a discussion of observations and experiments made by other investigators on migration in various species of Teleosts. This review will not include all the work done in this field, because in recent years excellent surveys have been written by Fontaine and Koch (1950), Hoar (1951b, 1953), Black (1951), Fontaine (1946, 1948, 1953, and 1954), and Smith (1956). The present survey will only give the most important results obtained until now and show the advances achieved and difficulties met in the study of migration in fishes.

Pituitary activity and migration

Vilter (1946) noticed that normal elvers entering fresh water are strongly halophobic, but that hypophysectomized elvers are much less halophobic. Fontaine, Callamand, and Olivereau (1949) found that hypophysectomy did not affect the salinity tolerance of the adult eel. Pickford (1953a) showed that hypophysectomized *Fundulus* became much less euryhaline. Smith (1956) found that pituitary extracts raised the salinity tolerance in brown trout. Woodman (1939) was unable to find differences between the pituitaries of parr and smolts (of Atlantic Salmon), but Fontaine and Oliverau (1949), working with the same species, observed fewer basophilic cells in the pituitary of smolts than of parr (basophilic cells are the producers of gonadotrophic and thyrotrophic hormones).

Changes in osmotic regulation at the time of migration and sexual maturity

The first investigators to emphasize the rôle of osmotic regulation in fish were Fontaine and Callamand (1940), Koch (1942), and Fontaine (1943, 1946). They also were aware of the possible rôle of the thyroid gland in the causation of migration.

Fontaine and Callamand (1940, 1941, 1943, and 1948), and Callamand and Fontaine (1940) found in the silver eel at the time of migration a considerable reduction in the amount of chlorides present in the body fluids. Koch (1949) on the other hand was unable to show a noticeable difference in the amount of chlorides at the time the gonads started to develop, i.e. in migrating eels. This controversy, however, might have been caused by the fact that the investigators studied the above problem at different times of the year. In migrating

elvers the osmotic control is disturbed at the time of metamorphosis, i.e. when the active migration towards the coast begins (VILTER, 1946; FONTAINE and CALLAMAND, 1941). The changes in osmotic regulation of the two species of Sticklebacks (*Gasterosteus* and *Pygosteus*) at the time of gonadal maturation and spring migration have been discussed on p. 284. In the Atlantic Salmon the fry and parr (non-migrating phases) are not able to withstand a direct transfer to salt water (HUNTSMAN and HOAR, 1940; HUNTSMAN, 1945). However, the migrating form, the smolt, is able to tolerate a direct transfer to sea water (HUNTSMAN, 1945; VIBERT, 1948). There is more variation on this subject among the different species of Pacific Salmon, but the general conclusion is that here too the smolts are no longer adapted physiologically to fresh water (HOAR and BELL, 1950).

Thyroid activity at the time of migration

The elver and eel show active thyroid glands at the time of migration (CALLAMAND and FONTAINE, 1942; FONTAINE and CALLAMAND, 1943; BERNARDI, 1948), but according to VILTER (1944) many elvers entering fresh water show quiescent glands. However, at the beginning of migration, i.e. at metamorphosis, the thyroid gland is always active (MURR and SKLOWER, 1928; SKLOWER, 1930; VON HAGEN, 1936; FRANÇOIS, 1941). Elvers entering fresh water show strong positive rheotropism. After administration of thiourea their rheotropism was much less marked (FONTAINE, 1948). In the Atlantic Salmon the thyroid gland is active at the time of parr-smolt transformation, i.e. at the time of the beginning of seaward migration (FONTAINE and OLIVEREAU, 1947; HOAR, 1939; FONTAINE, 1948). In the rainbow trout the thyroid is active when the smolts enter the lakes from the rivers (ROBERTSON, 1948). Sticklebacks have an active thyroid gland at the time of spring migration (KOCH and HEUTS, 1942). In the Pacific Salmon some species migrate with quiescent glands, while others have more active thyroids at that time (HOAR and BELL, 1950). HOAR, MCKINNON and REDLICH (1952), and HOAR, KEENLEYSIDE, and GOODALL (1955) obtained an increase in the rate of swimming and in sensitivity of young salmon to external stimuli after administration of thyroxine. The adult Atlantic Salmon shows an active thyroid gland on entering the rivers (FONTAINE and OLIVEREAU, 1947; FONTAINE, LACHIVER, LELOUP and OLIVEREAU, 1948; FONTAINE and LELOUP, 1950a, b). The thyroid gland is also active in adult *Alosa alosa* entering rivers (FONTAINE and LELOUP, 1950a).

Thyroid hormone and osmotic regulation

KOCH and HEUTS (1942), and HEUTS (1943) were able to show that

in the Stickleback species *Gasterosteus* and *Pygosteus* living in sea water the feeding of thyroid substance caused a reduced capacity for osmotic regulation. This did not happen when they were living in fresh water. SMITH (1956) found that injection of thyroxine in brown trout (non-migratory species) raised the salinity tolerance, whereas administration of thiourea and thiouracil reduced this tolerance. HOAR (1951b) observed that immersion of young coho salmon in a thyroxine solution for two or three days did not affect their salinity tolerance.

The general conclusion that can be drawn from this survey is that in most cases there is a connection between changes in osmotic regulation, pituitary and thyroid activity, general activity of the fish and migration. However, when an attempt is made to build up a detailed picture of the way in which these phenomena are connected one meets with great difficulties.

In the first place it has been suggested that the increased activity of the thyroid gland at the time of migration might be a secondary effect. HOAR and BELL (1950), and HOAR (1952) found that when fish of salmon species that usually migrate to the sea are retained in fresh water after the time of migration, they showed hyperactive thyroid glands. The authors interpret this phenomenon as a metabolic response to counteract the stress of the abnormal osmotic conditions. OLIVEREAU (1948) and LELOUP (1948) found that when stenohaline marine fish were transferred to water with a lower degree of salinity, this resulted in an increased activity of the thyroid gland. When stenohaline fresh water fish were transferred to water with a higher salinity the thyroid gland showed a fall in activity (OLIVEREAU, 1950, 1954; SMITH, 1956). It must be emphasized that the above fish were stenohaline and thus did not belong to species that migrate between the sea and fresh water. It is possible that the reaction of migratory species to the above conditions would have been different, while, moreover, the time of the year in which the experiments were made have not been taken into account.

The experiments with Sticklebacks in which thyroxine induced a change in salinity preference and also disturbed the osmotic equilibrium indicates that in this case the activity of the thyroid gland in spring is most probably not a secondary effect, but a primary cause of the above phenomena and thus of migration. It is possible that the primary thyroid activity (caused by various stimuli) and the secondary activity (caused by residence in the medium that has become inappropriate) may act together in inducing migratory behaviour (see also FONTAINE, 1954).

In the second place the observations and experiments mentioned

in the above survey have shown that some species of fish have active thyroid glands at the time of migration to fresh water, while most probably this gland is hypoactive at the time of seaward migration. In other species of fish the thyroid gland seems to be active at the time of migration to fresh water as well as at the time of seaward migration. This makes it difficult to conceive the mechanism of the effect of thyroid hormone on osmotic regulation and/or on migratory behaviour (see section 4c). However, it is very well possible that the mechanism inducing migration in the different species has become adapted, each in its own way, to changes in thyroid activity.

It is also possible that the fact that some species of fish have active thyroids both at the time of seaward migration and of migration to fresh water (eel, salmon) must be explained by assuming that the mechanism inducing migration in immature (or young) and maturing fish (adults) of the same species is affected differently by thyroid activity. It is clear that more data on the activity of the thyroid gland in migratory species are badly needed.

Though it is very likely that the thyroid gland and possibly also osmotic regulation are involved in the causation of migration in a number of teleost fish, it is at the moment impossible to decide whether this conception can be generally applied.

It is possible that many conflicting data have resulted from the experimental method used to study different phenomena and by other factors, some of which will be mentioned below.

1. It seems as if many investigators have not realised sufficiently that the responses of non-migratory animals to changes in the external condition might be different from the responses of migratory species. This also holds for the effect of administration of different hormones etc. To give an example, in birds it is known that exposure of migratory and resident birds of the same species to extra light in the spring results in a deposition of fat reserves in the migratory birds (indication of preparation for migration), whereas this response does not occur at all in the resident birds (Wolfson, 1942, 1945). Fontaine (1954) has pointed out that fluctuations in the activity of the thyroid gland are larger in migratory than in sedentary species of fish. This also holds for the rate of activation of the thyroid gland which is greater in the former than in the latter species. Thus, more fruitful work can be done if the above mentioned difference between the responses of migratory and sedentary species are taken into account. Migration and its accompanying phenomena should first be studied in migratory species. The study of this phenomenon in resident species can only be useful by giving data for comparison, but the latter may never be used as a basis for the explanation of migration.

2. Experiments on salinity tolerance and preference and the effect of hormones on these phenomena should be made at different times of the year, because the physiological condition of the animals varies between the different seasons. Further, the temperature at which the experiments are made should be taken into account since it may influence the response of the animals to changes in salinity (HEUTS, 1945) and may also influence the activity of the thyroid gland (see Chapter IX).

3. Methods for studying the activity of the thyroid gland should be made more uniform. Both histological and chemical or physical examinations should be carried out at the same time, since it is known that the different methods may reveal opposite results (OLIVEREAU, 1955c; FONTAINE, 1948).

4. It has been shown that in fishes the histological aspect of the thyroid gland varies in relation to the season (VON HAGEN, 1936; LIEBER, 1936; HOAR, 1939; BUCHMANN, 1940; BARRINGTON and MATTY, 1954, and SWIFT, 1955), the degree of maturation (BERNARDI, 1948; SWIFT, 1955) and various other factors such as metamorphosis and diet (see HOAR, 1951b). Therefore it will be difficult to ascertain whether changes in activity of the thyroid gland are related to migration or whether they have been caused by other factors acting at the same time.

If future work on the phenomenon of migration in fishes would take the above considerations into account, more conclusive data could be obtained that would allow a better explanation of the causation of migration.

In the survey only the effect of anterior pituitary and thyroid hormones on migration have been considered, but as has already been mentioned earlier, it is possible that other hormones or mechanisms may equally well be involved. One must keep in mind that though it has been conclusively shown that at the time of migration profound changes take place in the osmotic system of some Teleosts, there is as yet no proof that these changes are the *causal* factors inducing actual migration.

However, let us assume for a moment that the change in osmotic regulation does play a rôle in migration. It has been shown that administration of thyroid hormone causes a profound change in the osmotic regulation of the Stickleback. However, other hormones might also be able to induce such a change. In mammals it is known that the adrenal cortex plays an important rôle in the salt balance of the body fluids. In fish the interrenals are homologous with the adrenal cortex and it is possible that this organ may be involved in hydro-mineral metabolism and thus in osmotic regulation. Work on this subject has just started (see FONTAINE, 1956). Corticosteroids may also

be involved in the causation of migration for FONTAINE and HATEY (1954; see FONTAINE, 1956) were able to show that the corticosteroid value was higher in smolts than in parr. The medullo-suprarenal hormone adrenalin is able to stop the active chloride secretion of eels in sea water (KEYS and BATEMAN, 1932) and therefore might also play a rôle. The posterior lobe of the pituitary may also be involved in hydro-mineral metabolism of fishes by secreting two substances known as ADS (antidiuretic substance) and WBP (waterbalance principle) (see FONTAINE, 1956).

Apart from hormones it is known that the nervous system may play a rôle in osmotic regulation, for DRILHON (1942) found that section of the medulla reduced the salinity tolerance of the carp.

This survey shows that it cannot yet be concluded that thyroid hormone is the only factor involved in the causation of changes in osmotic regulation.

On the basis of the considerations given in this and the previous section it is evident that no definite conclusion can be made on the causation of migration in fishes. With respect to migration in the three-spined Stickleback it seems that among the factors studied so far the pituitary-thyroid mechanism, influenced by external and intrinsic factors is one of the most important mechanisms involved in the causation of migration.

IX. THE INFLUENCE OF RISING TEMPERATURE ON SALINITY PREFERENCE AT DIFFERENT TIMES OF THE YEAR

In homoiothermic animals it is known that the thyroid gland becomes more active when the temperature is lowered (see BUDDENBROCK, 1950; OLIVEREAU, 1955c). This increased activity is conceivable since the animals have to counteract the greater loss of heat by increasing their metabolic activity.

In cold blooded animals general activity depends mainly on the temperature of the medium, the animals becoming sluggish at low temperatures. Therefore, in cold blooded animals an increased activity of the thyroid gland at temperatures below a certain level cannot be expected. However, it is possible that fluctuations in body temperature above that level might be regulated by the activity of the thyroid gland in that an increase in environmental temperature may induce a greater activity of the gland and thus stimulate the animals to greater activity.

However, experiments on this subject have revealed a number of

contradictory results which will be discussed at the end of this chapter. In the Stickleback the problem was especially interesting since in nature the animals seem to migrate in larger numbers as soon as the temperature rises suddenly in the early months of the year (see Chapter II). It was decided to perform a number of experiments in which the fish were exposed to increasing temperatures at different times in the year.

I. EXPERIMENTS AND RESULTS

The salinity preference was studied in different groups of fish that had been caught in their natural habitats.

Experiment 43. The fish had been caught in a ditch on November 1, 1955. They were under-yearling animals and measured about 3.5 cm. The temperature of the water in the ditch at that time was about 5° C. The fish were transported to the laboratory and tested immediately after arrival. Fig. 22 shows that the animals preferred salt water. After the test they were placed in an aquarium in which the temperature gradually rose from 10° to 14° C. When they were tested 24 hours later they still showed a salt water preference. Five days later the preference was again for salt water. The day length during the experiment was the natural day length at that time of the year (about 9–10 hours).

This result shows that a raise in temperature from 5° to 14° C and maintenance of five days at the latter temperature does not affect the salinity preference at this time of the year.

Experiment 44. The animals used were adult fish that had been caught in the sea on December 16, 1954. The temperature of the water at that time was about 5° C. The fish were transported to the laboratory and placed in a big tank with a temperature of about 12° C. They were tested within 16 hours after arrival and showed a predominant preference for salt water, though they preferred fresh water in a number of sub-tests (see fig. 22). The fish were maintained at the same temperature while the day length was the natural day length at that time of the year, namely 8 hours. When the fish were tested 24 hours later they had no preference and another 24 hours later they showed a prevailing preference for fresh water, though they preferred salt water in one sub-test and had no preference in a number of other sub-tests.

In this case it seems as if the sudden increase in temperature from 5° to 12° C had caused a change in salinity preference from salt to fresh water, though the latter was not consistent. However, it is

possible that the change would have later become definite if the experiment had been continued longer.

Experiment 45. Under-yearling fish were used that had been maintained in the basin outside the laboratory from the time of hatching in April and May. They were caught on January 12 of the next year when the temperature of the water was 5° C. The fish were tested immediately after capture and showed a prevailing salt water preference (see fig. 22). They were then placed in an aquarium of about 10° C in which the temperature gradually rose to 16° C during the night. When they were tested 24 hours after the first test; they had no preference and another 24 hours later they preferred fresh water. Thus at this time of the year an increase in temperature from 5° to 16° C results in a change in preference from salt to fresh water.

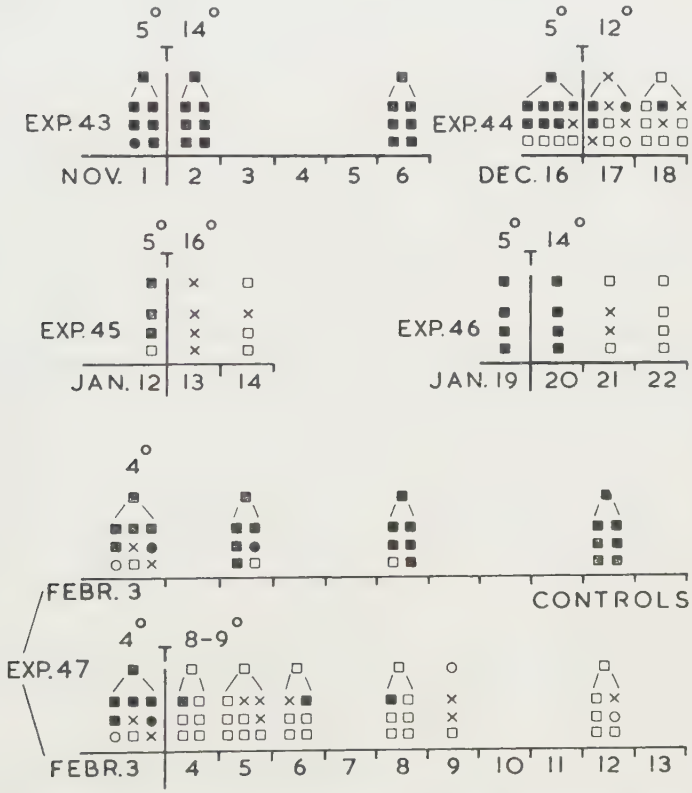

Fig. 22. Influence on salinity preference of a rise in temperature at various times of the year. T = date of transfer to water with a higher temperature. For further explanation see fig. 12.

Experiment 46. Adult fish were used that had been kept in the basin outside the laboratory from April. On January 19 of the next year they were tested immediately after capture and showed a salt water preference (see fig. 22). The temperature of the water was 5° C. After this test the fish were kept in an aquarium in which the temperature rose to 14° C during the night. When they were tested the next day they still preferred salt water. However, another 24 hours later they had changed their preference, though not yet consistently. On the third day after capture they showed a consistent fresh water preference.

Thus it follows again that in the first month of the year a rise in temperature from 5° to 14° C results in a change in preference from salt to fresh water.

It was a pity that in none of the experiments described above controls were kept under the initial low temperature in order to determine whether or not the preference in these fish would have remained the same during the experiment. Though this seems to be very likely, it was nevertheless decided to carry out another experiment in which controls were kept at the initial low temperature.

Experiment 47. The animals used were adult fish that had been caught in brackish water near Den Helder on February 2. At that time the temperature of the water was about 5° to 6° C. The animals had been stored at Den Helder for a couple of days in running cold sea water. After transportation to the laboratory they were kept in a big tank outside the building where the temperature was about 4° C. Some of the fish were kept in the aquarium-house where the temperature varied between 8° and 9° C. When the fish were tested immediately after arrival in the laboratory they had a predominant preference for salt water, as can be seen in fig. 22.

The fish kept at 8° to 9° C were tested 1, 2, 3, 5, 6, and 10 days after arrival. It can be seen that right after the rise in temperature the fish changed their preference and preferred fresh water. This lasted for the next ten days when the experiment was terminated. The fresh water preference was not always consistent, but the Pt values were below the 0.01 level in all cases but one.

However, the controls that had been kept at the initial low temperature all the time did not change their preference, but maintained a preference for salt water.

This experiment shows conclusively that in February a rise in temperature from 4° to 9° C results in a change in preference from salt to fresh water.

The conclusion that can be drawn from the above experiments is that

it depends on the time of year whether or not the Sticklebacks will respond to a rise in temperature by changing their salt water preference to one for fresh water. They do not respond in November, but do so in the early months of the year, while in December there may already be a tendency to change the preference. In Part I it has been shown that the animals are in different physiological phases at different times in the season and, therefore, it is very likely that the physiological condition of the fish determines whether or not it will respond to a rise in temperature.

This phenomenon may be of great importance for the timing of the onset of migration. It seems that the animals will remain in the sea as long as the temperatures are low during the first months of the year (i.e. about 4° to 5° C). At that time the pituitary is most probably not yet able to respond to the weak stimulus of the external conditions by secreting a sufficient amount of thyrotrophic hormone and thus to induce a sufficient output of thyroid hormone to cause a fresh water preference. It is also possible that the low temperature directly inhibits the secretion of thyroid hormone. However, as soon as the temperature increases the secretion of thyroid hormone is suddenly increased (either by a greater production of thyrotrophin or by an increased activity of the thyroid). The result is that the salt water preference changes to a fresh water preference within a few days. This sudden change in salinity preference may be the cause of the increase in numbers of migrating Sticklebacks after a sudden rise in temperature (see Chapter II).

However, in case the temperature remains low during the first three months of the year (which happened in 1955 and 1956) this does not mean that the fish will not migrate. For in this period of time the stimulus of the external condition, particularly of the day length, is gradually becoming stronger so that later in the season the pituitary will be able to secrete a sufficient amount of thyrotrophic hormone even in the absence of a rise in temperature. This assumption is supported by the fact that in 1956 the migration of Sticklebacks started as soon as the ice began to melt in March, i.e. when the water temperature varied between 0° and 4° C.

The fact that the fish do not respond to an increase in temperature in autumn is also important, for it prevents a migration to fresh water at an inappropriate time of the year.

In the above experiments it has been shown that in the early months of the year a rise in temperature changes the salinity preference and thus the disposition for migration. However, in this case it is also possible that the rise in temperature at the same time stimulated the migration-drive, for the fact that the number of migrating Sticklebacks

increases after a sudden rise in temperature can also be explained by assuming that the temperature affected the migration-drive (under the influence of which the animals actually move) in animals already being in a disposition to migrate (see p. 219).

No experiments were done on the influence of a decrease in temperature at the end of the breeding season, but it would certainly be interesting to know whether or not such a change would result in a change in preference from fresh to salt water. An indication that a decrease in temperature might affect the salinity preference can be obtained from the observation that when autumn is mild the arrival of the Sticklebacks in the sea is later than when the temperature has been low (see Chapter II).

2. DISCUSSION

The results of the above experiments showing that in spring a rise in temperature causes a change in salinity preference from salt to fresh water are in full agreement with the results of HEUTS' experiments (1945). This investigator demonstrated that the effectiveness of osmotic regulation in Sticklebacks in salt water decreased with increasing temperatures. Since it was known that feeding of thyroid substance also caused a reduced osmotic regulation (KOCH and HEUTS, 1942; HEUTS, 1943) of Sticklebacks in sea water, it is very likely that temperature affects the output of thyroid hormone. It is also possible that the effect of this hormone becomes greater at higher temperatures (see review of LYNN and WACHOWSKY, 1951).

With regard to the above statement that a rise in temperature might affect the migration-drive in the three-spined Stickleback, the experiments of KEENLEYSIDE and HOAR (1954) with young salmon are of interest. They found that an increase in temperature above 10° C induced a negative rheotaxis in fish which are positively rheotactic at temperatures below 10° C. They also mentioned observations of other workers who had shown that the response of adult salmon to currents may also be affected by changes in temperature. Rheotactic responses are thought to be involved in the causation of migration of young salmon (HOAR, 1951a, b, 1953, 1956).

A large number of investigators working with cold blooded animals have demonstrated an increase in the activity of the thyroid gland as a consequence of a rise in temperature, whereas others were either unable to show an increased activity under these conditions or even found a decrease (see reviews by LYNN and WACHOWSKY, 1951, and OLIVEREAU, 1955c). In the following a short survey of the results of experiments on this subject made in fishes will be given.

OLIVEREAU (1950, 1955a, b, c, d) working with different species of elasmobranch and teleost fishes did not observe an increase in activity of the thyroid gland as a consequence of a rise in temperature. She used histological methods in the majority of experiments and both histological and physical methods (radio-active iodine) in other experiments.

On the other hand an increase in activity of the thyroid gland as a result of an increase in temperature was reported by BLANC and BUSER (1949), BARRINGTON and MATTY (1954), and FORTUNE (1955). All these investigators used histological methods.

It is difficult to account for these contradictory results, but it is possible that at least part of the contradiction has been caused by the different methods used to determine the activity of the thyroid gland. Since it is very difficult, if not impossible, to deduce from the histological picture of a gland whether or not it is *secreting* hormones, the best method for determination of activity (i.e. secretion) of the thyroid gland is the determination of the rate of turn-over of radio-active iodine by the gland, or the determination of the amount of iodine in the blood.

Another fact that may have caused part of the contradictory results is that most experiments were carried out in a rather limited period of the year in which some of the species used might not be sensitive to temperature stimulation.

Cyclical changes in the activity of the thyroid gland have been shown by several authors whose names have already been mentioned on p. 287. The assumption that the thyroid may show cyclical changes in sensitiveness to external factors such as temperature is supported by personal experiments with Sticklebacks described in this chapter, which indicated that the thyroid gland does not respond to a rise in temperature in autumn, while it does respond in the early months of the year.

Finally it is possible that the temperature range that was used to determine the effect of a rise in temperature on the activity of the thyroid gland in certain species of fish was not appropriate for this study. It is possible that in one species thyroid activity is adapted to a change in temperature from 0° -5° C, for example, and in another species to a change from 10°–15° C. This would mean that if the activity of the thyroid gland of the former species had been studied at a temperature between 10° and 15° C no response could be expected, while it would have reacted at a temperature between 0° and 5° C. In the same way a study of the latter species at a temperature between 0° and 5° C would not have given a response, while it would have been obtained if the study was made at a temperature between 10° and 15° C. In

the Stickleback it seems that the critical temperature range in spring lies somewhere between 4–5° and 8–9° C (see section 1 of this chapter).

Studies on the activity of the thyroid gland under various external conditions are badly needed, particularly in migratory species.

X. CONCLUSIONS

The experiments described in the previous chapters have demonstrated a number of facts that will now be used to build a theory on the causation of migration in the three-spined Stickleback. The orientation during migration and the termination of migration will be briefly discussed at the end.

In the theory it will be left undecided whether thyroid hormone affects the central nervous system directly, or whether this occurs indirectly by disturbance of the osmotic equilibrium (see section 4c, Chapter VIII).

In the description of the theory a number of marks will be used to indicate on what kind of observations and experiments the statements are based. The explanation of the marks is as follows: ◆, facts and conclusions obtained in the present study; ○, ditto obtained by other investigators who have studied the same species; *, ditto obtained in other species of fish; and △, assumptions usually supported by some evidence.

The majority of Sticklebacks winter in the sea or brackish water (◆, ○). During winter the day lengths are short and the temperatures low. This condition is unfavourable for the secretion of thyroid hormone and the animals have a preference for salt water (◆).

In the early months of the year the day lengths are increasing and this stimulates the secretion of both gonadotrophic and thyrotrophic hormones (◆) and possibly also of other hormones (△). If the temperature remains low during January the secretion of gonadotrophins is only slightly hampered (◆) but the output of thyroid hormone is still low and the animals maintain their salt water preference (◆). However, if the temperature rises in January the secretion of thyroid hormone becomes sufficient to cause a disturbance of the osmotic equilibrium (○) and a change in preference from salt to fresh water (◆), which means that the fish have come into migration-disposition. In case the temperature also remains low in February and March a high temperature is no longer necessary to cause a sufficient secretion of thyroid hormone, for at this time the day length has become long enough to stimulate a sufficient activity in the thyroid (◆, ○) to bring the fish in migration-disposition (◆). There is no intrinsic factor, which in the absence of

certain external factors causes the change in preference from salt to fresh water.

During the early months of the year the secretion of thyroid hormone is stimulated also by the fact that the developing gonads already produce small amounts of gonadal hormones (◆). However, the gonads are not essential for the induction of migration-disposition (◆, *), though they may influence migratory activity and influence the sensitivity of the fish to external stimuli (*).

At the time of the increased activity of the thyroid gland (○) the animals migrate to fresh water under the influence of certain external factors causing the migration-drive (△).

When the animals arrive in fresh water they start to breed as soon as the external conditions induce maturation of the gonads (◆). The fresh water preference remains as long as secretion of thyroid hormone is above a certain level (◆). However, during the breeding period the high concentration of gonadal hormones present in the blood somewhat inhibits the secretion of thyroid hormone and consequently the fish show either a weak fresh or salt water preference (◆). In nature this tendency towards a salt water preference during the breeding period does not induce migration because it is too weak and because the external conditions are not favourable for seaward migration (no induction of migration-drive (△).

The activity of the pituitary in secreting gonadotrophic and thyrotrophic hormones (or the secretion of gonadal and thyroid hormones) is terminated by the co-operation between external and intrinsic factors (◆). At the time when the secretion of thyroid hormone has dropped below a certain level the fish start to prefer salt water (◆) and migrate seaward. The latter may take place immediately after the breeding season has ended (○), or later when the external conditions induce migration-drive (△). When the fish arrive in the sea or in brackish water the cycle is closed.

Under-yearling fish prefer salt water when they are about two months old (◆) and probably even earlier. At this time the secretion of thyroid hormone is rather low and the result is that they show a salt water preference. These animals may start to migrate seaward already in mid-summer (○), but it is also possible that they start to migrate seaward as soon as the external conditions have induced the migration-drive (△). Some of the fish may stay in fresh water (◆) though they show a salt water preference (◆). In the early months of the year they respond to external conditions in the same way as the adults.

So far this detailed picture of the causation of migration in the three-spined Stickleback. No work has been done yet on orientation during

migration and on the factors terminating migration, but a few remarks on these subjects will be made below.

FONTAINE and KOCH (1950) suggested that the gradient of salinity that is present in most coastal waters, rivers and even lakes might be a guide for orientation during migration. Such a mechanism for orientation is difficult to conceive, but it is possible that, while swimming, the animals constantly "check" whether the medium fits their internal physiological condition or not. This may be done in the same way as they probably do during the salinity tests. It implies that the fish must be able to discriminate between very small differences in salinity degrees. KRINNER (1934), and BULL (1937) have shown that some species of fish are able to discriminate between 34 pro mille, and 33·94 or 33·55 pro mille. *Phoxinus* appeared to be 184 times more sensitive to a NaCl concentration than man. It seems, therefore, that the suggestion of FONTAINE and KOCH on the method of orientation during migration might be true.

However, it is also possible that other external factors may serve as guides, e.g. currents, light and smell of the water. Studies on the orientation of salmon have shown that in these fish the smell of the parent-stream enables them to return to the streams where they hatched (HASLER and WISBY, 1951; HASLER, 1954). HOAR (1956) found that salmon fry can rapidly learn fixed directions of swimming and it is possible that this mechanism is involved in orientation during migration. Finally it is possible that fishes possess a "sense of direction" or, like birds, are able to use the sun in navigation (KRAMER, 1951, 1952, and 1953; MATTHEWS, 1955).

Factors that are responsible for the termination of migration seem never to have been studied. In the case of the Stickleback it is possible that seaward migration ends as soon as the fish have reached the sea or brackish water and that thereafter they merely roam about until spring migration is induced. However, the above problem is certainly more complicated in the case of spring migration. Some Sticklebacks evidently stop migrating as soon as they have entered fresh water, for breeding fish can be found in ditches and other shallow places only a short distance inland. However, other animals travel 30 km or more before they start breeding. Are the ones that travel the shortest distance the first to start breeding and do they thus occupy territories that others have to pass before being able to settle for breeding? Or is there a certain amount of "migration energy" that has to be consumed before migration is ended? The latter would imply that the amount of energy is different in all animals or in groups of fish. It may also be possible that recognition of the breeding place plays a rôle and that the

fish, both adults and young, return to the place where they have bred or hatched. The answer is unknown yet and its solving seems to be one of the most difficult problems in the study of migration.

Though the above picture on the causation of migration in the three-spined Stickleback is for the most part based on experiments and is probably largely accurate, the discussions at the end of the different chapters have shown that much future work has to be done before a conclusive statement can be made. However, the detailed description of the way in which migration may be caused in the Stickleback makes the phenomenon more conceivable and offers a firm basis for future research, even if it would later appear that the explanations or interpretations have been wrong on a number of points.

From what has been said in Chapter VIII, it is clear that the above theory in general follows that of FONTAINE and KOCH (1950), FONTAINE (1954), and KOCH (1955). However, the present theory has a firmer basis and shows a more detailed aspect.

The theories agree in that migration is caused by changes in the neuro-endocrine system, particularly the hypophyso-thyroid mechanism. These changes induce the particular behaviour pattern known as migration. The theories also agree in that they deny that gonadal hormones would play a primary rôle in the causation of migration, though the present investigation gave evidence that gonadal hormones may have a modifying effect.

FONTAINE has been right in emphasizing the fact that before migration can start internal changes must have taken place. However, it is not clear from his statements whether he thinks that these internal changes are intrinsic or whether they are caused by external factors. The present investigation has shown the primary rôle of external factors in inducing the internal changes as the time of spring migration (migration-disposition) and the joint rôles of intrinsic and external factors in inducing the changes at the time of seaward migration. However, it will always be difficult to ascribe a primary rôle to some factor (or to groups of factors) because of the delicate interrelations that exist between the organism and its environment.

Once the internal change has taken place migration starts as soon as the external factors are appropriate for induction of the migration-drive. The theories of FONTAINE and KOCH and that of the present author also agree on this point. These external factors may be similar to those affecting the neuro-endocrine system and causing migration-disposition, but may also be quite different.

The behaviour of Sticklebacks before and after the induction of migration in nature is not known, but it seems likely that it changes

at the time of migration, because the fish have to travel great distances and must be prepared to surmount obstacles that separate them from their goal. It is possible that they are much more active and alert during the migratory period and have become susceptible to external factors to which they were indifferent before.

This conception agrees with that of HOAR (1951b, 1953) who believes that in diadromous fish the endocrine changes that take place at the time of migration affect the behaviour of the fish and thus causes the onset of migration. HOAR also believes that gonadal hormones are in some way involved in migration, for he and his pupils (see p. 252) were able to show that these hormones affected the general activity of the fish and their sensitivity to external stimuli. However, as has been argued before their experiments are not conclusive on this point, since the gonadal hormones may have acted by stimulating the production of thyroid hormone.

HOAR, on the other hand is not convinced that changes in thyroid activity or in osmotic regulation are possible primary causes of migration, because not all migrating species show such changes at the time of migration. However, it is possible that in each species of fish the mechanism responsible for the induction of migration has become adapted in its own way to external and internal changes, and may even have become adjusted to different factors. Besides, it has been argued before that the different methods used to study thyroid activity are not all equally reliable and sometimes have revealed contradictory results.

WOLFSON (1942, 1945, 1947, and 1952), who studied migration in birds, also holds that the gonads are not essential for the induction of migration, though the basis of this assumption in birds is not so sound as in the Stickleback. WOLFSON has evidence that the increasing day lengths in winter and spring induce a change in the physiological condition of the birds, which ultimately causes the onset of migration. This internal change is indicated by a heavy deposition of fat (WOLFSON, 1945, 1952, 1954a, b; SCHILDMACHER, 1952, 1955). This fat deposition is influenced by the photo-period (WOLFSON, 1947; SCHILDMACHER, 1955, 1956), by gonadal hormones (SCHILDMACHER and STEUBING, 1952; RAUTENBERG, 1952/1953), and by thyroxine (SCHILDMACHER and RAUTENBERG, 1952). WAGNER (1930, 1956) found that thyroid hormone and gonadal hormones increased the restlessness in migratory birds kept in cages. MERCKEL (1938) also found that thyroid hormone increased restlessness. It is striking that also in birds the hypophyso-thyroid system seems to be involved in the causation of migration.

All theories on migration in birds and fishes agree in the assumption that migration is caused by externally and internally regulated neuro-

endocrine changes. However, this is still a rather vague concept and many variations in the details of its elaboration in various groups can be imagined. It remains for future work to decide how many of these possibilities are realised in nature and hence to decide whether a more detailed general theory of the causes of migration can be drawn up.

XI. SUMMARY

Chapter I. The methods and ways of approach used in studies on the causation of migration—i.e. the periodical displacement between two areas—are discussed. A survey is given of different viewpoints on this subject that can be found in the literature. In this way the design is developed of the present experiments that are primarily directed to study the rôle of external (day length; temperature) and internal factors (pituitary; gonads; thyroid gland) and of their interaction in the causation of migration.

Chapter II. The material, methods and technique used in this study are described. Sticklebacks winter in the sea or in brackish water and migrate to fresh water in spring. At the end of the breeding season they migrate back to the sea.

Changes in salinity preference have been used as criterion for the induction of migration-disposition. The term disposition (or motivation) is used here in the following sense. It is thought that first an internal change takes place in the fish (caused by external and/or intrinsic factors) inducing a readiness to respond to the external factors releasing the actual migration movements. This readiness is called disposition. The migration movements are caused by the migration-drive. The word drive is used in the sense of "the total quantity of information supplying a behaviour". The choice of salinity preference as criterion for migration-disposition is justified in Chapter IV.

The general procedure of the experiments was to subject the animals to an experimental treatment and then to test their salinity preference. Consequently a great deal of these tests were carried out with fish used in experiments described in Part I.

Chapter III. A number of experiments are described in which the influence on salinity preference was determined of various unwanted but unavoidable factors present in most salinity tests.

Chapter IV. The salinity preference of animals living under natural conditions was tested at various times of the year. In the early months of the year adult fish prefer fresh water, whereas shortly after the end of the breeding season they show a salt water preference.

Young fish hatched in April and May prefer salt water as early as about two months after hatching and have a preference for fresh water in the early months of the next year, just as the adults. This result of the salinity tests is in full agreement with observations on migration made in nature and justifies the choice of salinity preference as criterion for migration disposition.

Chapter V. Animals in different phases of gonadal development were exposed to various external conditions in order to obtain information on the effect of such conditions on gonadal development and salinity preference.

The results show that the attainment of maturity is always accompanied by a change in preference from salt to fresh water. However, in some cases in which no complete maturation took place, a change in preference from salt to fresh water nevertheless occurred, though a less prominent one than when full maturity is attained. It is possible that in these cases a slight development of the gonads had yet taken place, which then would have correlated with the change in salinity preference.

In experiments in which gonadal development was inhibited at a very early phase (phase 0 or the beginning of 1a) no change in preference from salt to fresh water took place, not even when the fish were maintained under the same constant condition for over one year. This result indicates that there is no intrinsic factor that is able to induce a change in preference from salt to fresh water under this constant external condition which also inhibits the attainment of maturity.

Animals kept for long periods of time at a long day length combined with a high temperature show alternating reproductive and non-reproductive periods and, correspondingly, they also show alternating periods of fresh and salt water preference. This does not occur at a short day length combined with a high temperature.

In the course of the reproductive period the fresh water preference becomes less prominent, but the definite change in preference from fresh to salt water takes place only after the end of the breeding phase.

It can be concluded from the above that, like the end of the reproductive period, the change in preference from fresh to salt water is also brought about by an interaction between intrinsic and external factors.

All experiments demonstrate that gonadal development is closely correlated with a change in preference from salt to fresh water, whereas gonadal regression is correlated with a change in preference from fresh to salt water. However, it is evident that no conclusion can be drawn on the ground of the above experiments as to whether there is a *causal* relation between the two phenomena.

Chapter VI. In order to decide whether or not there is a causal relation between the gonadal cycle and salinity preference, gonadectomized fish were exposed to various external conditions known to affect the development or regression of the gonads in normal fish.

The gonadectomized animals reacted to the external conditions in the same way as the normal unoperated fish. This result shows that the gonads do not play a primary rôle in the causation of changes in salinity preference and thus in the induction of migration-disposition.

Evidence was obtained that gonadal hormones may have a modifying effect on the timing of the changes in salinity preference. It is suggested that the changes in salinity preference are caused by a hormone, the production of which is influenced by the amount of gonadal hormones present in the blood.

Chapter VII. Animals were treated with mammalian gonadotrophic hormones in order to investigate the influence of these hormones on salinity preference. In fish with relatively far developed gonads administration of gonadotrophin resulted in a precocious maturation of the gonads and also caused a premature change in preference from salt to fresh water. However, both effects only appeared a rather long time after the beginning of the treatment (about 3 weeks).

Gonadotrophic hormones were also administered to fish subjected in phase 0 to a short day length combined with a high temperature. In these fish maturation stayed away nor did the initial salt water preference change. However, the hormones did induce a very weak tendency towards a change in preference from salt to fresh water. Again the effect was achieved rather a long time after the beginning of the treatment (about 4 weeks).

In both cases it is likely that the gonadotrophins had stimulated the secretion of gonadal hormones, which in their turn stimulated the output of the hormone controlling salinity preference. The results indicate that gonadotrophic hormones do not play a primary rôle in the causation of migration.

Chapter VIII. Since several investigations on migration in fish suggest a relation between this phenomenon and the activity of the thyroid gland a number of experiments were made to determine the effect of thyroxine and thiourea (a thyroid inhibitor) on salinity preference.

It is shown that thyroxine induces a fresh water preference in animals initially preferring salt water, whereas thiourea induces a salt water preference in fish initially preferring fresh water. In both instances the change in preference took place within 3–5 days after the beginning of the treatment. There is evidence that this effect is not or only very little influenced by external conditions.

The results also show that administration of thiourea to gonadectomized fish preferring fresh water results in a change in preference from fresh to salt water, just as in unoperated animals.

From all experiments it can be concluded that the amount of thyroid hormone present in the blood determines whether the fish will prefer fresh or salt water. The gonads may only have a modifying influence by affecting the secretion of thyroid hormone.

In a separate section the mechanism of the hormonal influence on salinity preference is discussed. It is suggested that the salinity preference owes its cyclical character either to changes in the osmotic regulation system (possibly influenced by thyroid hormone), which in their turn would affect the central nervous system, or by a change in the latter system caused by other stimuli (either hormonal—including the thyroid hormone—or nervous) than those coming from the osmotic mechanism.

Finally a survey is given of the existant data on the correlation found in many migratory species of fish between migration on the one hand and on the other hand pituitary activity, osmotic regulation and thyroid activity. Though the data agree on a number of points, many contradictory results have been found. It is argued that part of the latter may have been due to the diversity in the methods used to study migration and its accompanying phenomena. Several factors that might have caused confusion in work in this field are mentioned.

It is concluded that the hypophyso-thyroid mechanism is undoubtedly involved in the causation of migration, though other factors may also play a rôle.

Chapter IX. The effect is described of a rise in temperature on salinity preference at various times in the year. In November a rise in temperature from 5° to 14° C followed by maintenance at the latter temperature did not affect the salt water preference of the Sticklebacks. However, in the early months of the year a similar rise in temperature induced a fresh water preference in animals initially preferring salt water. The change took place within 1–3 days after the rise in temperature. It is very likely that the temperature affected the hypophyso-thyroid mechanism. The conclusion is that it depends on the time of the year, and thus on the physiological condition of the fish, whether or not they will respond in the above way to a rise in temperature.

This phenomenon might prevent an untimely migration to fresh water in autumn or early winter after a sudden rise in temperature.

The possible effect of temperature on thyroid activity is discussed in the light of data from the literature.

Chapter X. A theory is presented on the causation of migration in the

three-spined Stickleback. In this theory migration is thought to be caused by a delicate interaction between environmental and intrinsic factors. Changes in the hypophyso-thyroid mechanism, due to external as well as intrinsic factors, are thought to be the primary cause of migration. Gonadal hormones do not play an essential rôle in this process, though they may have a modifying influence.

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REFERENCES

BAERENDS, G. P., BROUWER, R. and WATERBOLK, H. TJ., 1955: Ethological studies on *Lebistes reticulatus* (Peters). I. An analysis of the male courtship pattern. Behaviour, **8**, 249–334.

BAKER, J. R. and BAKER, I., 1936: The seasons in a tropical rain-forest (New Hebrides). Part II. Botany. J. Linn. Soc. (Zool.) Lond., **39**, 507–519.

BARGMANN, W., 1954: Das Zwischenhirn-Hypophysensystem. Springer Verlag Berlin.

BARRINGTON, E. J. W. and MATTY, A. J., 1954: Seasonal variation in the thyroid gland of the Minnow, *Phoxinus phoxinus* L., with some observations on the effect of temperature. Proc. Zool. Soc. Lond., **129**, 89–95.

BEACH, F. A., 1948: Hormones and behaviour. Hoeber Inc. N. York.

BEDFORD, DUKE OF and MARSHALL, F. H. A., 1942: On the incidence of the breeding season in mammals after transference to a new latitude. Proc. Roy. Soc. Lond. B., **130**, 396–399.

BENOIT, J. and ASSENMACHER, I., 1953a: Rapport entre la stimulation sexuelle préhypophysaire et la neurosécrétion chez l'oiseau. Arch. Anat. microsc. et Morph. expérim., **42**, 334–386.

FONTAINE, M., 1953: La fonction hypophyso-thyroidienne des poissons dans les rapports avec leur morphologie et leur comportement. J. du Conseil, **19**, 23–38.

FONTAINE, M., 1954: Du déterminisme physiologique des migrations. Biol. Rev., **29**, 390–418.

FONTAINE, M., 1956: The hormonal control of water and salt electrolyte metabolism. Mem. Soc. Endocrinol., **5**, 69–82.

FONTAINE, M. and CALLAMAND, O., 1940: Influence de la température sur l'élimination chlorée de l'Anguille. C. R. Ac. Sci. Paris, **211**, 488–489.

FONTAINE, M. and CALLAMAND, O., 1941: Sur certaines facteurs des migrations de l'Anguille. Bull. Soc. Zool. Fr., **66**, 68–76.

FONTAINE, M. and CALLAMAND, O., 1943: Les aspects physiologiques d'une "vie cyclique de l'Anguille d'Europe", *Anguilla anguilla*. Bull. Mus. Hist. Nat., **15**, 373–378.

FONTAINE, M. and CALLAMAND, O., 1948: Nouvelles recherches sur le déterminisme physiologique de l'avalaison des poissons migrateurs amphibiotiques. Bull. Mus. Hist. Nat., **20**, 317–320.

FONTAINE, M., CALLAMAND, O. and OLIVEREAU, M., 1949: Hypophyse et euryhalinité chez l'Anguille. C. R. Ac. Sci. Paris, **228**, 513–514.

FONTAINE, M. and KOCH, H. J., 1950: Les variations d'euryhalinité et d'osmorégulation chez les poissons. J. Physiol., **42**, 287–318.

FONTAINE, M., LACHIVER, F., LELOUP, J. and OLIVEREAU, M., 1948: La fonction thyroidienne du Saumon (*Salmo salar*) au cours de sa migration reproductrice. J. Physiol., **40**, 182–184.

FONTAINE, M. and LELOUP, J., 1950a: Sur l'iodémie de deux téléostéens migrateurs potamotoques, *Salmo salar* L. et *Alosa alosa* L., au debut de leur montée reproductrice. C. R. Ac. Sci. Paris, **230**, 775–777.

FONTAINE, M. and LELOUP, J., 1950b: L'iodémie du Saumon (*Salmo salar* L.) au cours de sa migration reproductrice. C. R. Ac. Sci. Paris, **230**, 1216–1218.

FONTAINE, M. and OLIVEREAU, M., 1947: La glande thryoide du Saumon atlantique (*Salmo salar* L. ♀) au cours de sa vie en eau douce. C. R. Ac. Sci. Paris, **224**, 1660–1662.

FONTAINE, M. and OLIVEREAU, M., 1949: L'hypophyse du Saumon à diverses étapes de sa migration. C. R. Ac. Sci. Paris, **228**, 772–774.

FORTUNE, P. Y., 1953: Thyroid activity in Teleosts. Nature, **171**, 483–484.

FORTUNE, P. Y., 1955: Comparative studies of the thyroid function in Teleosts of tropical and temperate habitats. J. exp. Biol., **32**, 504–513.

FRANÇOIS, Y., 1941: Evolution de la thyroide chez les civelles. C. R. trav. Fac. Sci. Marseille, **1**, 74–77.

GIERSBERG, H. and STADIE, R., 1934: Ueber experimentelle Auslösung des Zugtriebes durch weibliches Sexualhormon. Vogelzug, **5**, 173–176.

GOLDSMITH, E. D., NIGRELLI, R. F., GORDON, A. S., CHARIPPER, H. A. and GORDON, M., 1944: Effect of thiourea on fish development. Endocrinol., **35**, 132–134.

GREELEY, F. and MEYER, R. K., 1953: Seasonal variation in testis-stimulating activity of male pheasant pituitary glands. Auk, **70**, 350.

GROEBBELS, F., 1928: Zur Physiologie des Vogelzuges. Verhdl. Ornith. Gesell. Bayern, **18**, 44–74.

GROS CLARK, W. E. LE, MCKEOWN, T. and ZUCKERMAN, S., 1938: Visual pathways concerned in gonadal stimulation in ferrets. Proc. Roy. Soc. Lond. B, **126**, 449–468.

HAGEN, F. VON, 1936: Die wichtigsten Endokrinen des Flussaals. Thyroidea, Thymus und Hypophyse im Lebenszyklus des Flussaals (*A. vulgaris*) nebst eigenen Untersuchungen über das chromophile und chromophobe Kolloid der Thyroidea. Zool. Jhbr. Abt. Anat. u. Ontog., **61**, 467–538.

HANN, H. W., 1939: The relation of castration to migration in birds. Bird Banding, **10**, 122–123.

HARRINGTON, R. W., 1950: Preseasonal breeding by the Bridled Shiner, *Notropus bifrenatus*, induced under light-temperature control. Copeia, **4**, 304–310.

HARRIS, G. W., 1955: Neural control of the pituitary gland. Monogr. Physiol. Soc., **3**.

HASLER, A. D., 1954: Odour perception and orientation in fishes. J. Fish. Res. Bd. Canada, **11**, 107–129.

HASLER, A. D., MEYER, R. K. and FIELD, H., 1939: Spawning induced prematurely in trout with the aid of pituitary glands of the carp. Endocrinol., **25**, 978–983.

HASLER, A. D. and WISBY, W. J., 1950: Discrimination of stream odours by fishes and its relation to parent stream behaviour. Amer. Nat., **85**, 223–238.

HEUTS, M. J., 1943: La régulation osmotique chez l'Epinochette (*Pygosteus pungitius* L.). Ann. Soc. Zool. Belg., **74**, 99–105.

HEUTS, M. J., 1945: La régulation minérale en fonction de la température chez *Gasterosteus aculeatus* L. Ann. Soc. Zool. Belg., **76**, 88–99.

HEUTS, M. J., 1947a: The phenotypical variability of *Gasterosteus aculeatus* L. populations in Belgium. Meded. Kon. Vl. Akad. Wetensch., **9**, 5–63.

HEUTS, M. J., 1947b: Experimental studies on adaptive evolution in *Gasterosteus aculeatus* L. Evolution, **1**, 89–102.

HEUTS, M. J., 1949: Racial divergence in fin ray variation patterns in *Gasterosteus aculeatus* L. J. Genet., **49**, 183–191.

HIATT, R. W. and FISHER, H. I., 1947: The reproductive cycle of the ring-necked pheasants in Montana. Auk, **64**, 528–548.

HICKLING, C. F., 1935: Seasonal changes in the ovary of the immature Hake, *Merluccius merluccius* L. J. Mar. Biol. Ass. U. K., **20**, 443–462.

HILL, M. and PARKES, A. S., 1934: Effect of absence of light on the breeding season of the ferret. Proc. Roy. Soc. Lond. B, **115**, 14–17.

HOAR, W. S., 1939: The thyroid gland of the Atlantic Salmon. J. Morph., **65**, 257–296.

HOAR, W. S., 1951a: The behaviour of chum, pink and coho salmon in relation to their seaward migration. J. Fish. Res. Bd. Canada, **8**, 241–263.

HOAR, W. S., 1951b: Hormones in fish. Publ. Ont. Fish. Res. Lab., **71**, 1–51.

HOAR, W. S., 1952: Thyroid function in some anadromous and landlocked Teleosts (alewife and smelt). Trans. Roy. Soc. Canada, **46**, 39–53.

HOAR, W. S., 1953: Control and timing of fish migration. Biol. Rev., **28**, 437–453.

HOAR, W. S., 1955: Reproduction in teleost fish. Mem. Soc. Endocrin., **4**, 1–24.

HOAR, W. S., 1956: The behaviour of migrating pink and chum salmon fry. J. Fish. Res. Bd. Canada, **13**, 309–325.

HOAR, W. S. and BELL, G. M., 1950: The thyroid gland in relation to the seaward migration of Pacific Salmon. Can. J. Res., D, **28**, 126–136.

HOAR, W. S., KEENLEYSIDE, M. H. A. and GOODALL, R., 1955: The effects of thyroxine and gonadal steroids on the activity of Salmon and Goldfish. Canad. J. Zool., **33**, 428–439.

HOAR, W. S., MCKINNON, D. and REDLICH, A., 1952: Effects of some hormones on the behaviour of salmon fry. Canad. J. Zool., **30**, 273–286.

HÖHN, E. O., 1950: Physiology of the thyroid gland in birds: a review Ibis, **92**, 464–473.

HOOVER, E. E. and HUBBARD, H. E., 1937: Modification of the sexual cycle of trout by control of light. Copeia, **4**, 206–210.

HOPPER, A. F., 1950: The effect of mammalian thyroid powder and thiouracil on growth rates and on the differentiation of the gonopod in *Lebistes reticulatus*. Anat. Rec., **108**, 554.

HOPPER, A. F., 1952: Growth and maturation response of *Lebistes reticulatus* to treatment with mammalian thyroid powder and thiouracil. J. exp. Zool., **119**, 205–217.

HUNTSMAN, A. G., 1945: Migration of salmon parr. J. Fish. Res. Bd. Canada, **6**, 399–402.

HUNTSMAN, A. G. and HOAR, W. S., 1940: Resistence of Atlantic Salmon to sea water. J. Fish. Res. Bd. Canada, **4**, 409–411.

IERSEL, J. J. A. VAN, 1953: An analysis of the parental behaviour of the male three-spined Stickleback (*Gasterosteus aculeatus* L.). Behaviour, Suppl. **3**, 1–159.

IHERING, R. VON and AZEVEDO, P. DE, 1937: Ueber die Wirkung des Säugetier-Hypophysehormons auf den Laichakt der Fische. Zool. Anz., **120**, 71–75.

IKEDA, K., 1933: Effect of castration on the secondary sexual characters of anadromous three-spined Stickleback, *Gasterosteus aculeatus* L. Jap. J. Zool., **5**, 135–157.

JOHNSON, M. W. and RIDDLE, O., 1939: Tests of mammalian gonad-stimulating hormones on gonads of fishes. Proc. Soc. exp. Biol. Med., **42**, 260–262.

KEENLEYSIDE, M. H. A. and HOAR, W. S., 1954: Effects of temperature on the response of young salmon to water currents. Behaviour, **7**, 77–87.

KERR, T., 1948: The pituitary in normal and parasitized Roach (*Leuciscus rutilus* Flem.). Quart. J. Micr. Sci., **89**, 130–138.

KEYS, A. and BATEMAN, J. B., 1932: Branchial responses to adrenalin and to pitressin in the eel. Biol. Bull., **63**, 327–336.

KLUYVER, H. N., 1951: The population ecology of the Great Tit, *Parus m. major* L. Ardea, **39**, 1–135.

KLUYVER, H. N., 1952: Notes on body-weight and time of breeding in the Great Tit, *Parus m.major* L. Ardea, **40**, 123–141.

KNUDSEN, M., 1902: Berichte über die Konstantenbestimmungen zur Aufstellung der hydrographischen Tabellen. K. danske vidensk. Selsk. Math., **6**, 1.

KOCH, H. J., 1942: Cause physiologique possible des migrations des animaux aquatiques. Ann. Soc. Roy. Zool. Belg., **73**, 57–62.

KOCH, H. J., 1949: Quelques caractéristiques osmotiques de l'anguille femelle jaune et argentée. Arch. int. Physiol., **57**, 125–132.

KOCH, H. J., 1955: Au sujet d'un lien possible entre la physiologie et le comportement chez les poissons et les oiseaux migrateurs. La perception. Presse Univ. France, Paris, 121–128.

KOCH, H. J. and HEUTS, M. J., 1942: Influence de l'hormone thyroidienne sur la régulation osmotique chez *Gasterosteus aculeatus* L. forme *gymnurus*. Ann. Soc. Roy. Zool. Belg., **73**, 165–172.

KOCH, H. J. and HEUTS, M. J., 1943: Régulation osmotique, cycle sexuel et migration de reproduction chez les Epinoches. Arch. int. Physiol., **53**, 253–266.

KOCH, H. J. and HEUTS, M. J., 1944: Le cycle de la régulation osmotique et minérale du crabe chinois. Ann. Soc. Roy. Zool. Belg., **75**, 87–90.

KOCH, W. and SCHEURING, L., 1936: Die Wirkung von Hypophysenvorderlappenhormon auf den Laichakt von Fischen. Zool. Anz., **116**, 62–64.

KRAMER, G., 1951: Eine neue Methode zur Erforschung der Zugorientierung und die bisher damit erzielten Ergebnisse. Proc. 10th Int. Orn. Congr. Uppsala, 271–280.

KRAMER, G., 1952: Experiments on bird orientation. Ibis, **94**, 265–285.

KRAMER, G., 1953: Die Sonnenorientierung der Vögel. Verh. dtsch. Zool. Ges. Freiburg, 72–84.

KRINNER, M., 1934: Ueber die Geschmacksempfindlichkeit der Elritzen. Ztschr. vergl. Physiol., **21**, 317–342.

KROGH, A., 1939: Osmotic regulation in aquatic animals. Cambridge.

LEINER, M., 1930: Fortsetzung der ökologischen Studien an *Gasterosteus aculeatus* L. Ztschr. Morph. u. Oekol. d. Tiere, **16**, 499–540.

LEINER, M., 1931: Oekologisches von *Gasterosteus aculeatus* L. Zool. Anz., **93**, 317–333.

LELOUP, J., 1948: Influence d'un abaissement de salinité sur la cuprémie de deux téléostéens marins: *Muraena helena* et *Labrus bergylta* Asc. C. R. Soc. Biol. Paris, **142**, 178–179.

LEVER, J., MILTENBURG, J. and VAN OORDT, G. J., 1949: The effect of a short treatment with thiourea upon the fish thyroid gland. Proc. Kon. Nedl. Akad. Wetensch. A'dam, **52**, 296–300.

LIEBER, A., 1936: Der Jahreszyklus der Schilddrüse von *Misgurnis fossilis* und seine experimentelle Beeinflussbarkeit. Ztschr. wiss. Zool., **148**, 364–400.

LYNN, W. G. and WACHOWSKI, H. E., 1951: The thyroid gland and its functions in cold-blooded Vertebrates. Quart. Rev. Biol., **26**, 123–168.

MANN, H. B. and WHITNEY, D. R., 1947: On a test of whether one or two random variables is stochastically larger than the other. Ann. Mathem. Statist., **18**, 50–60.

MARSHALL, A. J., 1951: The refractory period of testis rhythm in birds and its possible bearing on breeding and migration. The Wilson Bull., **63**, 238–262.

MARSHALL, A. J., 1955: Reproduction in birds: the male. Mem. Soc. Endocrin., **4**, 75–93.

MARSHALL, F. H. A., 1936: Sexual periodicity and the causes which determine it. Phil. Trans. Roy. Soc. Lond. B., **226**, 423–456.

MARSHALL, F. H. A., 1937: On the change over in the oestrous cycle in animals after transference across the equator, with further observations on the incidence of the breeding seasons and the factors controlling sexual periodicity. Proc. Roy. Soc. Lond. B., **122**, 413–428.

MARSHALL, F. H. A, 1942: Exteroceptive factors in sexual periodicity. Biol. Rev., **17**, 68–89.

MARSHALL, F. H. A. and BOWDEN, F. P., 1934: The effect of irradiation with different wave-length on the oestrus cycle of the ferret, with remarks on the factors controlling sexual periodicity. J. exp. Biol., **11**, 409–422.

MATTHEWS, G. V. T., 1955: Bird navigation. Cambr. Monogr. Exp. Biol., **3**.

MATTHEWS, S. A., 1939a: The relationship between the pituitary gland and the gonads in *Fundulus*. Biol. Bull., **76**, 241–250.

MATTHEWS, S. A., 1939b: The effects of light and temperature on the male sexual cycle in *Fundulus*. Biol. Bull., **77**, 92–95.

MEDLEN, A. B., 1951: Preliminary observations on the effects of temperature and light upon reproduction in *Gambusia affinis*. Copeia, **2**, 148–152.

MEEK, A., 1916: The migration of fishes. London, Edw. Arnold.

MERCKEL, F. W., 1938: Zur Physiologie der Zugunruhe bei Vögeln. Ber. Ver. Schles. Ornith., **23**, 1–72.

MERRIMAN, D. and SCHEDL, H. P., 1941: The effects of light and temperature on gametogenesis in the 4-spined Stickleback (*Apeltes quadracus Mitchill*). J. exp. Zool., **88**, 413–446.

METUZALS, J., 1954: Neurohistologische Studien über die nervöse Verbindung der pars distalis der Hypophyse mit dem Hypothalamus auf dem Wege des Hypophysenstieles. Acta Anat., **20**, 258–285.

MEYERS, G. S., 1949: Usage of anadromous, catadromous and allied terms for migratory fishes. Copeia, **2**, 89–97.

MILLER, A. H., 1948: The refractory period in light-induced reproductive development of golden-crowned sparrows (*Zonotrichia coronata*). J. exp. Zool., **109**, 1–11.

MILLER, A. H., 1954: The occurrence and maintenance of the refractory period in crowned sparrows. Condor, **56**, 13–20.

MURR, E. and SKLOWER, A., 1928: Untersuchungen über die inkretorischen Organe der Fische. I. Das Verhalten der Schilddrüse in der Metamorphose des Aales. Ztschr. vergl. Physiol., **7**, 279–288.

NIGRELLI, R. F., GOLDSMITH, E. D. and CHARIPPER, H. A., 1946: Effects of mammalian thyroid powder on growth and maturation of thiourea-treated fish. Anat. Rec., **94**, 523.

NOWAKOWSKI, H., 1950: Zur Auslösung der Ovulation durch electrische Reizung des Hypothalamus beim Kaninchen und Ihre Beeinflussung durch Rückenmarkdurchschneidung. Acta Neuroveg., **1**, 13–39.

OLIVEREAU, M., 1948: Influence d'une diminution de salinité sur l'activité de la glande thyroide de deux Téléostéens marins (*Muraena helena* L. and *Labrus bergylta* Asc.). C. R. Soc. Biol. Paris, **142**, 176–177.

OLIVEREAU, M., 1950: Influence d'une augmentation de salinité sur l'activité thyroidienne de divers Téléostéens d'eau douce. C. R. Soc. Biol. Paris, **144**, 775–776.

OLIVEREAU, M., 1954: Hypophyse et glande thyroide chez les Poissons. Etude histophysiologique de quelques corrélations endocriniennes, en particulier chez Salmo salar. Ann. Inst. Océanogr. Monaco, **29**, 95–296.

OLIVEREAU, M., 1955a: Influence de la température sur le fonctionnement thyroidien de la Truite étudié à l'aide de l'iode radioactif. C. R. Soc. Biol. Paris, **149**, 536–539.

OLIVEREAU, M., 1955b: Influence de la température sur l'histologie thyroidienne de divers Téléostéens. Bull. Soc. Zool. France, **80**, 43–52.

OLIVEREAU, M., 1955c: Hormone thyréotrope et température chez la Truite arc-en-ciel (*Salmo gairdnerii*) et l'Anguille (*A. anguilla*). Arch. d'Anat. microsc. Morph. expér., **44**, 236–264.

OLIVEREAU, M., 1955d: Température et fonctionnement thyroidien chez les poissons. J. Physiol., **47**, 256–258.

OORDT, G. J. VAN and OORDT, P. G. W. J. VAN, 1955: The regulation of spermatogenesis in the frog. Mem. Soc. Endocrin., **4**, 25–38.

OORDT, P. G. W. J. VAN, 1956: Regulation of the spermatogenetic cycle of the common frog (*Rana temporaria*). Thesis, Utrecht.

OSTERHAGE, K. H., 1932: Morphologische und physiologische Studien an Pigmentzellen der Fische. Ztschr. mikr. anat. Forsch., **30**, 551–598.

OWEN, S. E., 1936: The reaction of fish to sex hormones. Endocrinol., **20**, 214–218.

PELKWIJK, J. J. TER and TINBERGEN, N., 1937: Eine Reizbiologische Analyse einiger Verhaltensweisen von *Gasterosteus aculeatus* L. Ztschr. f. Tierpsychol., **1**, 103–200.

PICKFORD, G. E., 1952: Induction of a spawning reflex in hypophysectomized Killifish. Nature, **170**, 807–808.

PICKFORD, G. E., 1953a: A study of the hypophysectomized male, Killifish (*Fundulus heteroclitus* L.). Bull. Bingham Ocean. Coll., **14**, 5–42.

PICKFORD, G. E., 1953b: The response of hypophysectomized male *Fundulus* to injections of purified beef growth hormone. Bull. Bingham Ocean Coll., **14**, 46–68.

PICKFORD, G. E. and PETRUNKEVITCH, A., 1955: Translation of paper by GERBILSKII (1951): Biological races of Caspian Sturgeons. Syst. Zool., **4**, 83–92.

PICKFORD, G. E. and THOMPSON, E. F., 1948: The effect of purified mammalian growth hormone on the Killifish (*Fundulus heteroclitus* L.) J. exp. Zool., **109**, 367–383.

PUTZIG, P., 1937: Von der Beziehung des Zugablaufs zum Inkretdrüsensystem. Vogelzug, **8**, 116–130.

PUTZIG, P., 1938: Weitere Versuche über die Beziehung der Keimdrüsen zum Zugverhalten. Vogelzug, **9**, 189–200.

PUTZIG, P., 1939: Keimdrüsen und Heimzug. Ber. Ver. Schles. Ornithol., **24**, 36–41.

RAMASWAMI, L. S. and HASLER, A. D., 1955: Hormones and sex characters in the Minnow, *Hyborhynchus*. Phys. Zool., **28**, 62–68.

RAUTENBERG, W., 1952/1953: Körpergewicht und Grundumsatz beim kastrierten männlichen Vogel. Wiss. Ztschr. Univ. Greifswald, **2**, 229–236.

RILEY, G. M., 1936: Light regulation of sexual activity in the male sparrow (*Passer domesticus*). Proc. Soc. Exp. Biol. Med., **34**, 331–332.

RILEY, G. M. and WITSCHI, E., 1938: Comparative effects of light stimulation and administration of gonadotrophic hormones on female sparrows. Endocrinol., **23**, 618–624.

ROBERTSON, O. H., 1948: The occurrence of increased activity of the thyroid gland in rainbow trout at the time of transformation from parr to silvery smolt. Physiol. Zool., **21**, 282–295.

ROWAN, W., 1929: Experiments in bird migration. I. Manipulation of the reproductive cycle; seasonal histological changes in the gonads. Proc. Bost. Soc. nat. Hist., **39**, 151–208.

ROWAN, W., 1930: Experiments in bird migration. II. Reversed migration. Proc. nat. Acad. Sci. Wash., **16**, 520–525.

ROWAN, W., 1932: Experiments in bird migration. III. The effects of artificial light, castration and certain extracts on the autumn movements of the American Crow (*Corvus brachyrhynchos*). Proc. nat. Acad. Sci. Wash., **18**, 639–654.

ROWAN, W., 1938: Light and seasonal reproduction in animals. Biol. Rev., **13**, 374–403.

ROWAN, W., 1946: Experiments in bird migration. Trans. Roy. Soc. Canada, **40**, 123–135.

SCHEURING, L., 1929: Die Wanderungen der Fische. I. Erg. d. Biol., **5**, 405–691.

SCHEURING, L., 1930: Die Wanderungen der Fische. II. Erg. d. Biol., **6**, 4–304.

SCHILDMACHER, H., 1933: Zur Physiologie des Zugtriebes. I. Vogelzug, **4**, 21–24.

SCHILDMACHER, H., 1934: Zur Physiologie des Zugtriebes. II. Vogelzug, **5**, 1–9.

SCHILDMACHER, H., 1937: Zur Physiologie des Zugtriebes. III. Vogelzug, **8**, 107–114.

SCHILDMACHER, H., 1938: Zur Physiologie des Zugtriebes. IV. Weitere Versuche mit künstlich veränderter Belichtungszeit. Vogelzug, **9**, 146–152.

SCHILDMACHER, H., 1939: Ueber die künstliche aktivierung der Hoden einiger Vogelarten im Herbst durch Belichtung und Vorderlappenhormon. Biol. Zentrbl., **59**, 653–637.

SCHILDMACHER, H., 1952: Stoffwechselphysiologische Studien an männlichen Gartenrotschwänzen, *Phoenicurus, ph. phoenicurus*. Biol. Zentrbl., **71**, 238–251.

SCHILDMACHER, H., 1955: Photoperiodizität des Stoffwechsels beim Vogel. Acta XI Congr. Int. Orn. Basel., 655–657.

SCHILDMACHER, H., 1956: Physiologische Untersuchungen am Grünfinken, *Chloris chloris* L., im künstlichem Kurztag und nach "hormonaler Sterilisierung". Biol. Zentrbl., **75**, 327–355.

SCHILDMACHER, H. and RAUTENBERG, W., 1952: Ueber die Wirkung kleiner Mengen von Thyroxin auf das Körpergewicht bei Finkenvögeln. Biol. Zentrbl., **71**, 397–405.

SCHILDMACHER, H. and STEUBING, H., 1952: Untersuchungen zur hormonalen Regulierung des Fettwerdens der Zugvögel im Frühjahr. Biol. Zentrbl., **71**, 272–282.

SCHREIBER, B., 1936: Ulterori osservazioni sugli elementi della linea germinale maschile in anguille maturate artificialmente con Prolan. Monit. Zool. Ital., **47**, Suppl., 197–200.

SCRUGGS, W. M., 1951: The epithelial components and their seasonal changes in the pituitary gland of the carp (*Cyprinus carpio* L.) and goldfish (*Carassius auratus* L.). J. Morph., **88**, 441–464.

SKLOWER, A., 1930: Die Bedeutung der Schilddrüse für die Metamorphose des Aales und der Plattfische. Forsch. u. Fortschr., **6**, 335–336.

SMITH, C. L., 1955: Reproduction in female Amphibia. Mem. Soc. Endocrin., **4**, 39–56.

SMITH, D. C. W., 1956: The rôle of the endocrine organs in the salinity tolerance of trout. Mem. Soc. Endocrinol., **5**, 83–101.

SMITH, H. W., 1932: Waterregulation and its evolution in the fishes. Quart. Rev. Biol., **7**, 1–26.

STADIE, R., 1938: Experimentelle Untersuchungen über den Wandertrieb gekäfigter Gartenrotschwänze, *Phoenicurus ph. phoenicurus*. Ber. Ver. Schles. Ornith., **23**, 65–80.

SWIFT, D. R., 1955: Seasonal variations in the growth rate, thyroid gland activity and food reserves of brown trout (*Salmo trutta* L.). J. exp. Biol., **32**, 751–764.

THOMSON, A. P. D. and ZUCKERMAN, S., 1954: The effect of pituitary-stalk section on light-induced oestrus in ferrets. Proc. Roy. Soc. Lond. B., **142**, 437–451.

TITSCHACK, E., 1923: Die sekundäre Geschlechtsmerkmale von *Gasterosteus aculeatus* L. Zool. Jahrb. Physiol., **39**, 83–148.

TURNER, C. L., 1919: The seasonal cycle in the spermary of the Perch. J. Morph., **32**, 681–711.

VAUGIEN, L., 1955: Sur les réactions testiculaires du jeune Moineau domestique illuminé à diverses époques de la mauvaise saison. Bull. Biol. France et Belg., **89**, 218–244.

VERHOEVEN, B. and VAN OORDT, G. J., 1955: The influence of light and temperature on the sexual cycle of the Bitterling, *Rhodeus amarus* Bl. Proc. Kon. Nedl. Akad. Wetsch. C., **58**, 628–634.

VERWEY, J., 1949: Migration in birds and fishes. Bijdr. t. d. Dierk., **28**, 477–504.

VIBERT, R., 1948: Travaux effectués en France sur le Saumon. J. du Cons., **3**, 328–333.

VILTER, V., 1944: Rhéotropisme de la Civelle et activité thyroidienne. C. R. Soc. Biol. Paris, **138**, 668–669.

VILTER, V., 1946: Déterminisme hypophysaire du comportement "halophobe" de civelles immigrantes. C. R. Soc. Biol. Paris, **140**, 483–484.

WAGNER, H. O., 1930: Ueber Jahres- und Tagesrhythmus bei Zugvögeln. Ztschr. vergl. Physiol., **12**, 703–724.

WAGNER, H. O., 1956: Die Bedeutung von Umweltfaktoren und Geschlechtshormonen für den Jahresrhythmus von Zugvögeln. Ztschr. vergl. Physiol., **38**, 355–369.

WILCOXON, F., 1945: Individual comparisons by ranking methods. Biometrics, **1**, 80–83.

WILHELMI, A. E., PICKFORD, G. E. and SAWYER, W. H., 1955: Initiation of the spawning reflex response in *Fundulus* by the administration of fish and mammalian neurohypophyseal preparations and synthetic oxytocin. Endocrinology, **57**, 243–252.

WOLFSON, A., 1942: Regulation of spring migration in Juncos. Condor, **44**, 237–265.

WOLFSON, A., 1945: The rôle of the pituitary, fat disposition and body weight in bird migration. Condor, **47**, 95–127.

WOLFSON, A., 1947: Fat deposition as a response to photoperiodism in migratory birds. Anat. Rec., **99**, 44.

WOLFSON, A., 1952: Day length, migration and breeding cycles in birds. Scient. Monthly, **74**, 191–200.

WOLFSON, A., 1954a: Weight and fat deposition in relation to spring migration in transient White-Throated Sparrows. Auk, **71**, 413–434.

WOLFSON, A., 1954b: Body weight and fat deposition in captive White-Throated Sparrows in relation to the mechanics of migration. Wilson Bull., **66**, 112–118.

WOODMAN, A. S., 1939: The pituitary gland of the Atlantic Salmon. J. Morph., **65**, 411–436.

WUNDER, W., 1928: Experimentelle Untersuchungen an Stichlingen. Zool. Anz., **3**, suppl., 115–127.

WUNDER, W., 1931: Experimentelle Erzeugung des Hochzeitkleides beim Bitterling (*Rhodeus amarus*) durch Einspritzung von Hormon. Ztschr. vergl. Physiol., **13**, 693–708.

WUNDER, W., 1934: Gattenwahlversuche bei Stichling und Bitterling. Verh. Deutsch. Zool. Ges., **36**, 152–158.

YEATES, N. T. M., 1947: Influence of variation in length of day upon the breeding season in sheep. Nature, **160**, 429–430.

SIZE FREQUENCY DISTRIBUTION OF SANDS IN THE DUTCH WADDEN SEA

A STUDY IN CONNECTION WITH ECOLOGICAL INVESTIGATIONS IN A TIDAL AREA

by

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I. INTRODUCTION

In 1932 the director of the Zoological Station, Dr. J. VERWEY, started an ecological survey of part of the fauna of the Dutch Wadden Sea near Den Helder. The investigations were followed up for a number of years and they are still continued to-day. In connection with the micro-distribution of the bottom animals bottom samples were also collected and the grainsize composition of these samples was determined. Up to 1955 about 2000 bottom samples were analysed.

Since it will probably take another series of years before the data relating to the distribution of the animals will be published, the data concerning the grainsize distribution of the sands are here published separately. Some grainsize figures have been used already in publications of KREGER (1940) and KUENEN (1942) and in a paper of KRISTENSEN (1957). Other aspects of this ecological research have been dealt with in papers of MAAS GEESTERANUS (1942), POSTMA and VERWEY (1950), POSTMA (1950, 1954), VERWEY (1952), and BAGGERMAN (1953).

The western Dutch Wadden Sea offers a very good opportunity for an investigation of the grainsize distribution of sandy deposits, since the bottom of this area mainly consists of sand and the sediments lie in widely different environments, from deep tidal channels to shoals emerging at low tide. The investigation comprises both the areal variations in grainsize and the grainsize composition of the individual

sand assemblages. The characteristics of the sand distribution will be explained on the basis of environmental conditions such as current velocity and wave action.

Investigations of this type are still comparatively scarce, though the study to be presented here is not the first of its kind carried out in Dutch coastal waters. Attention must be drawn to the investigations of VAN VEEN (1936), who studied sand movement in the Straits of Dover, along the Dutch coast and in the Dutch tidal inlets, in close relation with hydrographical conditions. In addition to this must be mentioned the work of DOEGLAS (1941, 1946, 1950) and his collaborators, who studied dune, beach and marine sands on the Dutch North Sea coast and sediments in the eastern Dutch Wadden Sea. In the latter investigations stress was laid especially on the influence of sediment mixing and differentiation and on the grainsize composition of the deposits.

Most of the samples have been taken in the Wadden Sea itself, but a considerable number, also to be discussed here, originate from the adjoining part of the North Sea. The geographical distribution of the sampling stations does not fully satisfy geomorphological requirements, but the material collected is sufficiently complete to outline the principal features of the sand distributions. It did not seem economical, however, to present all material available in full detail. For those who are interested, the complete data are on file at the Zoological Station.

The author himself participated in only a small part of the sampling cruises and therefore wants to express his thanks to all who contributed to the collection of the material. Thanks are especially due to Dr. J. VERWEY, director of the Zoological Station and Mr. I. KRISTENSEN, under whose supervision practically all samples were taken. Prof. Dr. D. J. DOEGLAS, Wageningen, Prof. Dr. PH. H. KUENEN, Groningen, and Dr. J. VERWEY haven given valuable comments on the contents of this paper. Mr. J. A. A. SPIEKERMAN, Den Helder, revised and corrected the English text.

II. THE AREA OF INVESTIGATION

The area studied, adjacent to the former Zuiderzee and since 1931 separated from the latter by a dike (fig. 1), can be described as a tidal area with passages into the North Sea. The tidal amplitude is about 1.5 m and the average depth some 3.5 m at H. W. About one third of the region consists of tidal flats ("Wadden"), separated from one another by tidal channels. In the passages to the North Sea (tidal inlets) the channels attain depths up to 35–50 m, whereas farther inward they are branched into narrower and shallower creeks and gullies.

The complicated morphology of the area is due to the action of

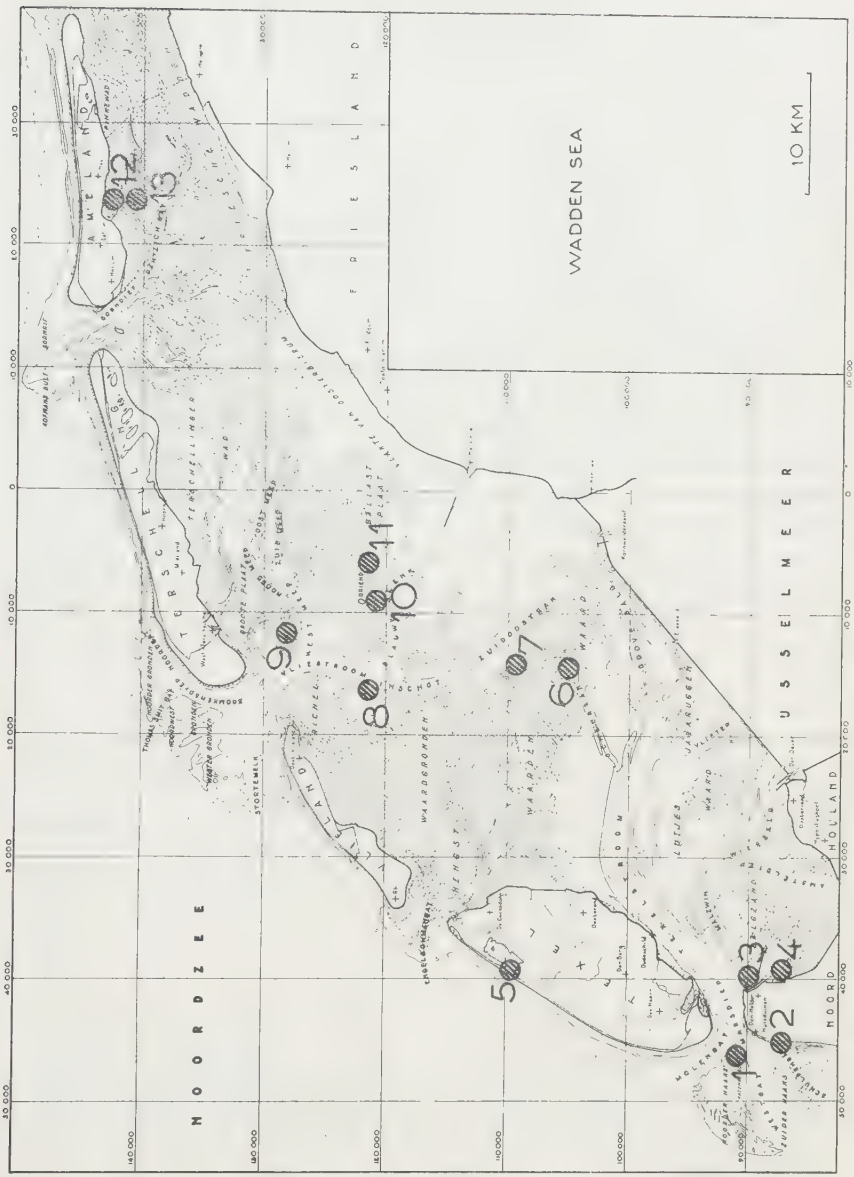


Fig. 1. The area of investigation from a chart of "Rijkswaterstaat". The dotted parts indicate tidal flats; the numbers indicate the location of tidal flats and beaches studied.

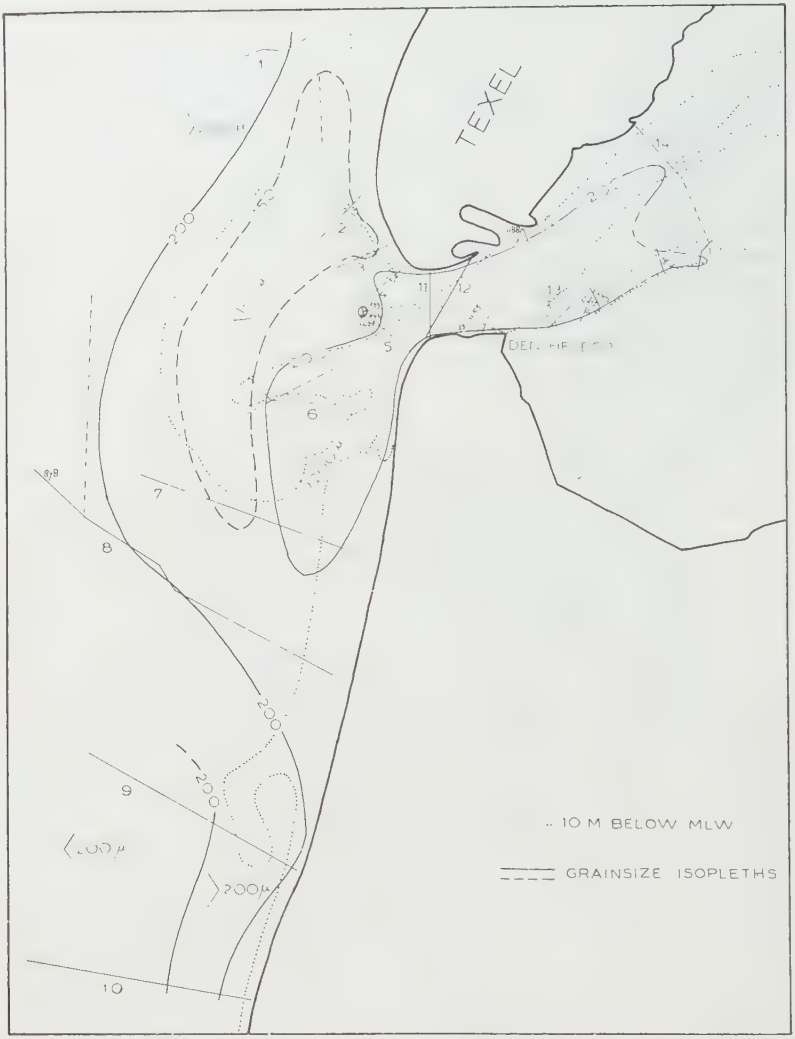


Fig. 2. Location of sampling traverses in and around the tidal inlet Marsdiep. Grainsize isopleths are approximative. The grainsize distribution along the full-drawn traverses is represented in figs. 3, 4 and 5.

strong tidal currents on a readily shifting bottom. Through the channels the sea water freely penetrates into the heart of the area and the filling and emptying of the tidal basin by ebb and flood thus meets with the least possible resistance. Strong currents of up to 200 cm/sec are normally found in the tidal inlets, whereas on the shoals velocities amount to only some 25 cm/sec. When tides and storm surges combine

much greater velocities occur. Ebb and flood carry on an average about the same amounts of water, so that no important residual transport of water takes place.

The greater part of the banks as well as of the creek bottoms consists of sand. On the flats the bottom is mostly hard and the sand very pure with occasional admixtures of shells or shell debris. Clay and organic matter are mostly found in only very small quantities, as in most places conditions are unsuitable for the deposition of fine material. Silty areas are only found on a few tidal flats well-sheltered against strong winds by the coast and in some deep channels artificially obstructed by dikes. The area investigated differs in this respect from the eastern part of the Dutch Wadden Sea, where the tidal flats are mostly covered with muddy sediments.—Many of the larger channels cut through old, often Tertiary, deposits and here the sands are often mixed with remains of old clay and peat beds, and pebbles. Especially in the tidal inlets large amounts of shell material are often encountered.

The samples collected in the North Sea were taken on and around the submarine shoals in front of the southernmost tidal inlet (Marsdiep; see figs. 1 and 2). Superimposed on the tidal streams we here find a residual current directed towards the North.

III. METHODS

All sampling cruises were made in the Station's small motor cutter "Max Weber", which, owing to its small size, made even very shallow locations easily accessible. The bottom samples from the tidal channels were taken by means of a large size grab designed by Van Veen (1936). This instrument, which seldom fails of its purpose, takes a sample over an area of 0.2 m^2 and 6–20 cm deep, the depth depending on the hardness of the bottom. The greater part of the grab contents was used for biological purposes; a small part of each sample was preserved for sand analyses. This subsample contained material from a surface layer of approximately 5 cm deep.—The samples from the tidal flats were taken at low tide, when the flats fall dry and are easy to explore.

The distribution of the sampling stations is given in figs. 1 and 2, and 7–13. It has already been remarked that from a geological point of view many samples were not taken systematically. For the most part such samples will not be considered here, as those collected along straight lines crossing channels and flats deliver sufficient material for the purpose of this study. The distances from station to station in the North Sea and the tidal channels vary between 100 and 500 m; stations on the tidal flats are mostly 50, sometimes 100 m apart.

In the laboratory the coarse material was separated from the sand

fraction by means of a 3 mm sieve. The material removed consisted of shell and peat debris and pieces of clay, sometimes small pebbles or gravel. The grainsize distribution of the samples was determined by means of the settling tube of VAN VEEN (1936). This method reveals the relation between the volume of material settled and the settling time. The grainsize was determined from the sinking velocity in sea water by means of tables, also published by VAN VEEN, which were obtained experimentally by comparing settling velocities with sieve analyses. The grainsizes do not exactly correspond with those calculated from settling velocity by the STOKES-SUDRY-formula, as the heavy material content of the sands was not taken into account (DOEGLAS, 1950). The results of the analyses are represented as cumulative grainsize distribution curves.

The above description shows that the samples did not undergo the treatment customarily applied in sedimentary analyses. No peroxide or hydrochloric acid was used to remove organic or calcareous material, and no attempt was made to disintegrate the comparatively coarse pellets in which the clay fraction is mainly present. This procedure has the disadvantage that comparison of our results with those of others may sometimes be difficult. On the other hand, a grainsize analysis of the bottom sediment as it is actually present in the sea may give a better insight into the relation between sediment composition and its environment.

The main fact about a sand sample that we can learn from the frequency distribution, is the median grainsize. The latter will be used in the next chapter to examine the grainsize distribution of the area. Obviously the frequency distribution as a whole presents a more complete picture of the properties of a sediment than the median by itself. The results of the complete grainsize analyses will be discussed separately in the last part of this paper (Chapter V).

IV. AREAL DISTRIBUTION OF MEDIAN GRAINSIZE

Among the factors which may be considered to contribute to the formation of bodies of sand of a certain grainsize, the intensity of water movement or turbulence will certainly play a prominent part. In a shallow area like the Wadden Sea turbulence may be caused by currents as well as by waves. A priori it seems reasonable to assume that wave action will especially be of influence in shallow areas, whereas currents will be of importance in tidal channels. To substantiate this supposition will be the main purpose of this chapter. Although a very large number of samples were examined, it was not found possible in general to draw isopleth maps of grainsize distribution. The main

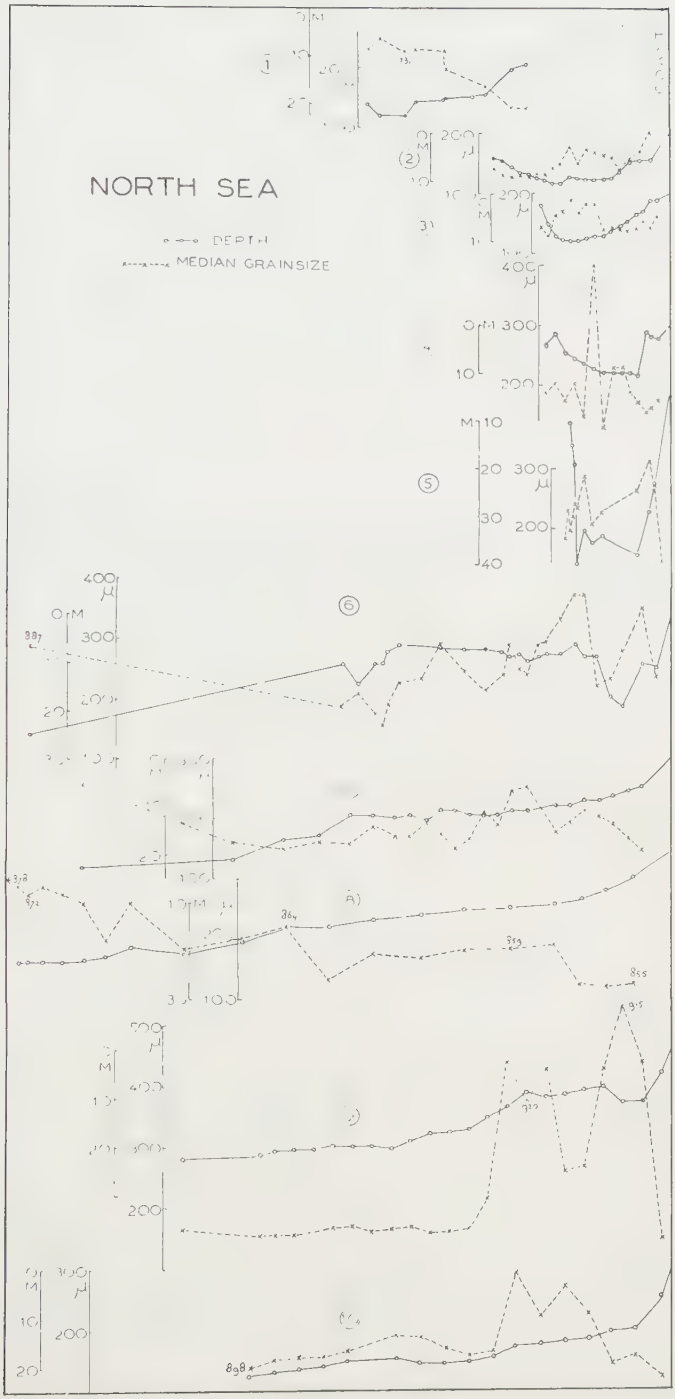


Fig. 3. Grainsize distribution along traverses of fig. 2; location of stations is only approximative.

difficulty was, that the majority of the samples were taken in the channels and that the series taken on the flats were mostly continued over too short distances. It was, therefore, necessary to give the results of the observations in the form of graphs representing series of samples along traverses. Only the distribution of the samples in the North Sea and the Marsdiep and of one tidal flat allowed of a geographical representation. We will first consider the grainsize distribution in the Wadden Sea itself and next discuss the distribution in the North Sea.

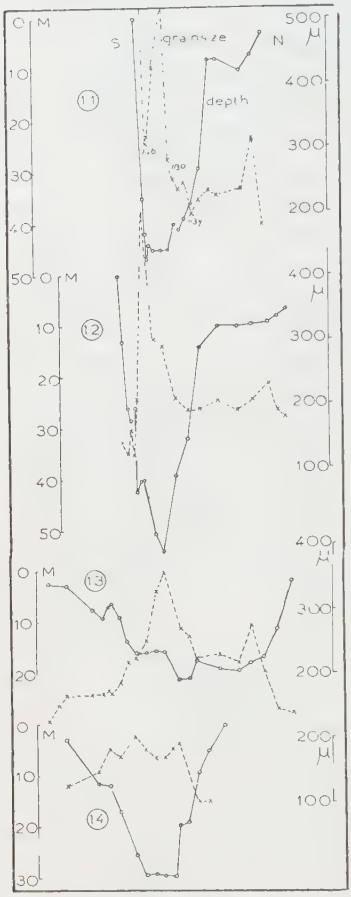


Fig. 4. Grainsize distribution along traverses of fig. 2; location of stations is only approximative.

A. WADDEN SEA

Examples of the grainsize distribution along traverses crossing channels are given in figs 3 (sections 2, 3, 4, and 5), 4, 5, and 6. Besides grainsize, the corresponding depth of every sample is represented. Since the position of the stations was not determined very accurately, the depth profiles are only approximative. The median grainsize appears to vary between about 100 μ and about 600 μ. Generally speaking, the coarsest material is found in the centre of the channels. Very coarse sediments occur in the tidal inlets. Since current velocities will attain their highest values in the deepest parts of the channels and especially in the passages to the North Sea, this distribution strongly suggests that current velocity is the main factor determining grainsize in the channels.

The coarsest material is not always found exactly in the deepest part of the channel. This may mostly be due to the fact that also the current maximum near the bottom does not always coincide with the centre of the channel. In general no exact equilibrium between bottom profile and current strength will exist, as is proved by the fact that most channels slowly change their position, become locally shallower owing to the deposition of material or are deepened by erosion. In some cases a different explanation may apply. We see for instance in section 17 of fig. 5 a

TABLE I

Average grainsize of tidal flats and beaches; for location see fig. 1

Nr.	Median grainsize	
	Md	Mø[1]
1	175 μ	2.5
2 (beach)	225	2.15
3	115	3.1
4	95	3.4
5 (beach)	210	2.25
6	100	3.3
7	120	3.05
8	100	3.3
9	190	2.4
10	125	3.0
11	110	3.2
12	110	3.2
13	115	3.1

[1] see p 339

regular increase of median grainsize from the sides of the channel towards the centre, but near the centre a sudden decrease of grainsize. The field description of the samples shows the presence in this area of much dumped

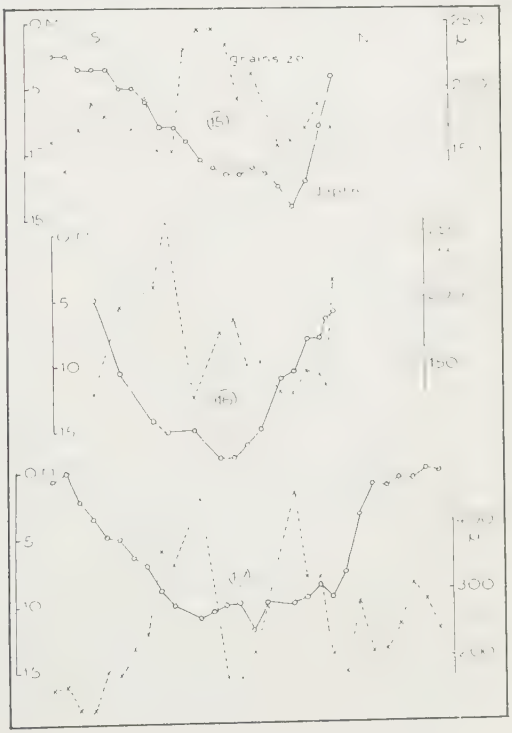


Fig. 5. Grainsize distribution along traverses of fig. 2; location of stations is only approximative.

or residual material, mostly large fragments of peat and clay, which serves as a shelter for fine sand grains.

The position of the tidal flats and beaches where samples were collected is given in fig. 1. Details of the topography of individual flats, together with the grainsize distribution along traverses crossing these flats are given in figs 7–13. Before considering these details we shall do well first to compare the flats among themselves. For this purpose table 1 summarizes the average median grainsizes.

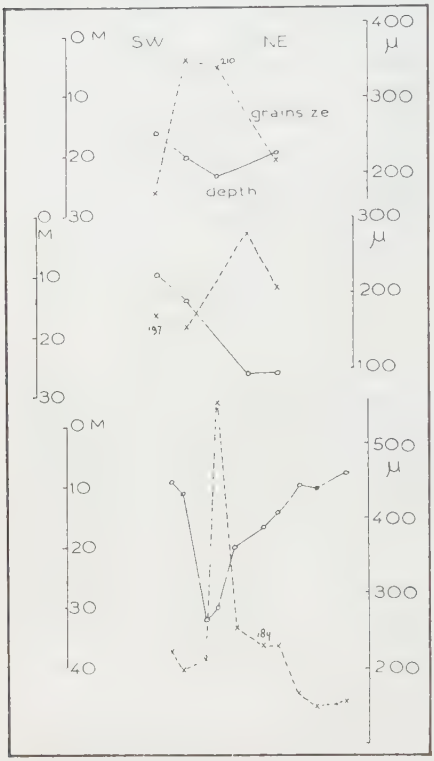


Fig. 6. Grainsize distribution along traverses of the tidal inlet between Vlieland and Terschelling (Vlie), see fig. 1; the distance between the traverses is about 1 km and the upper diagram indicates the northernmost cross-section.

The table shows the existence of considerable grainsize differences between the various flats. The cause of these differences becomes clear if we consider the geographical position of the flats as given in fig. 1. The largest grainsizes appear to occur on the flats directly exposed to the influence of the North Sea (1, 2, 5, and 9). Fine sands, on the other hand, are found especially in places sheltered on their west side by the coast (4); flats on the east side of a channel (7 and 10) are composed of coarser sands than those on the west side (6 and 8). It therefore seemsa logical assumption that these grainsize differences are not in the first place caused by differences in current velocity, but are primarily brought about by the action of waves, which are especially strongly developed in the North Sea. Moreover, wave influence will be greatest during stormy periods, when winds blow from the West.

The above assumption obtains further support from a consideration of the detailed grainsize distribution on the different flats. Extensive investigations were made on a tidal flat in the centre of the area studied (nr. 6 of fig. 1). The topography of this flat, together with the location of the sampling places and the positions of six stations where current

velocities were measured, is given in fig. 7. The full-drawn lines in this figure indicate the depth contours, whereas the dashed lines represent grainsize isopleths.

If first of all we consider the actual tidal flat, that is the area within the Mean Low Water line, we find that differences in average current velocity are only small. The complete current data, not reproduced

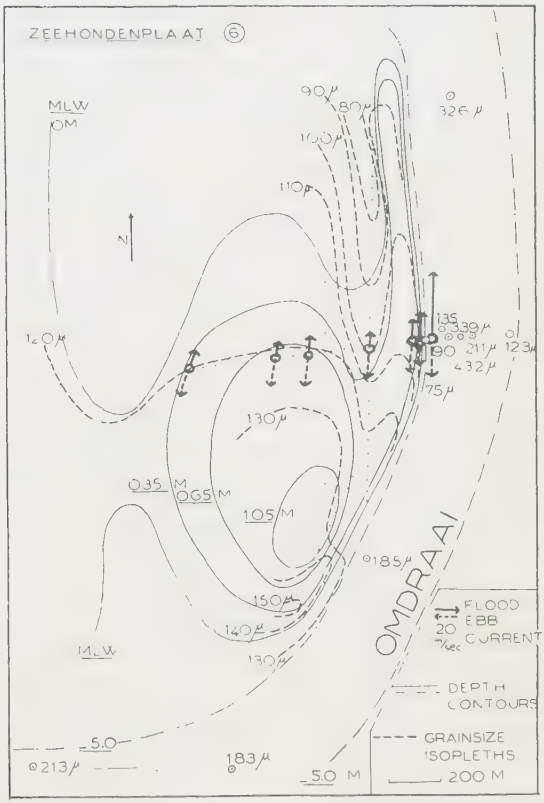


Fig. 7. The topography (full-drawn lines) of tidal flat nr. 6 (fig. 1) and its grainsize distribution (dashed lines). The arrows indicate average current velocity and direction during ebb and flood.

here, show that the same holds for current maxima. Therefore current strength cannot have played an important part in causing grainsize differences. Nevertheless the median grainsize varies between 80 and 150 μ, the coarsest sands occurring on the south side of the flat adjoining that part of the Omdraai channel, which isdirected towards the South-West (see fig.1). The finest material is found in a shallow, well-sheltered creek in the northern part of the bank. The whole distribution therefore again suggests that wave action is the dominant factor determining grainsize on the flat. The high grainsize values found in the adjoining tidal channel will again be caused by strong currents.

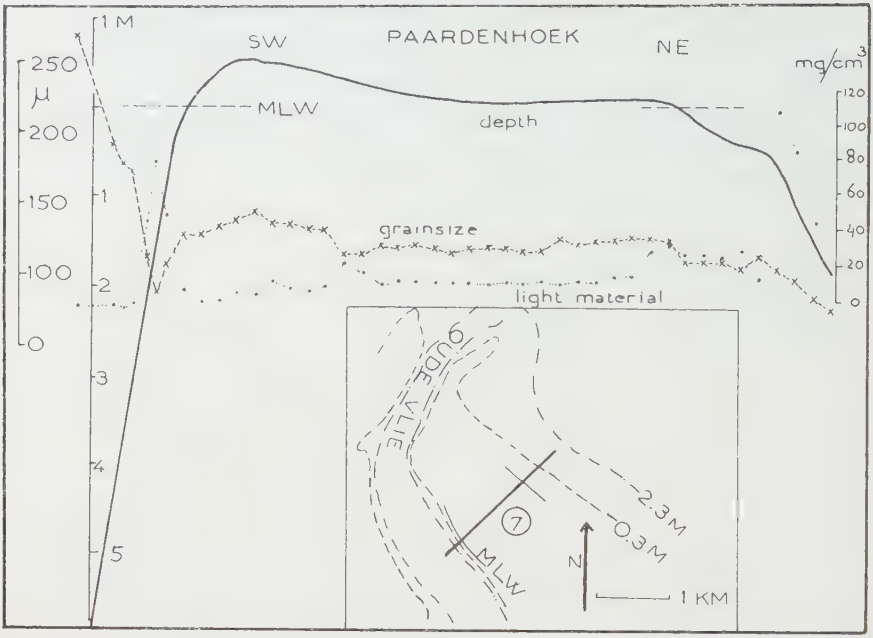


Fig. 8. Location, depth profile, grainsize distribution and amount of light material of the traverse of tidal flat nr. 7; compare fig. 1. Light material expressed in mg of dry weight per cm³ of sediment.

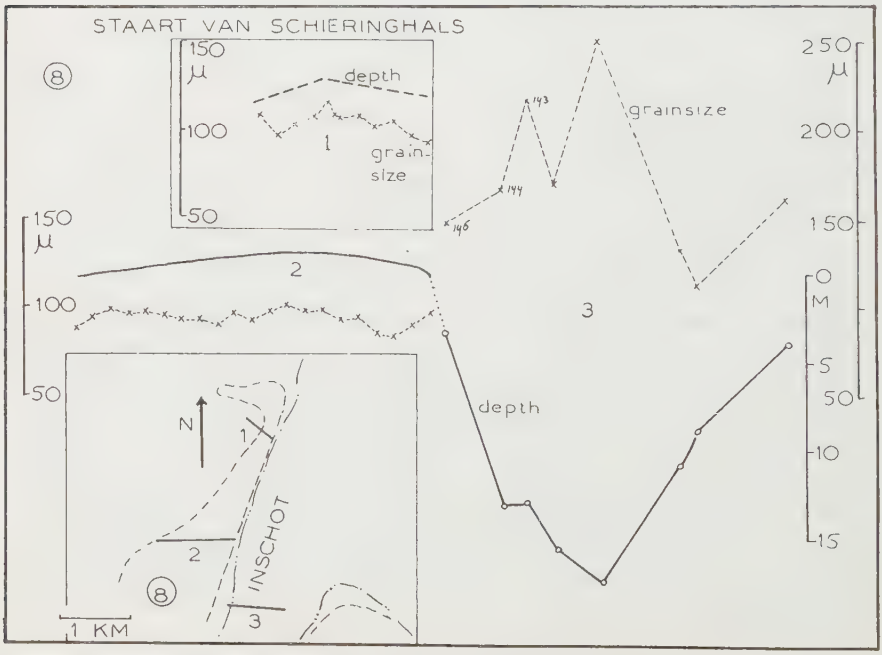


Fig. 9. Location, depth profiles and grainsize distribution of traverses of tidal flat nr. 8 and the adjoining Inschot channel; see fig. 1. Depth of section 1 is approximative.

Examination of the data collected on the other tidal flats confirms in general the point of view developed. The grainsizes along the cross-sections mostly show regular variations which can be explained by local differences in wave intensity. It must be realised, however, that in all these cases no current measurements are available, so that no certainty about the actual part played by currents on the various flats

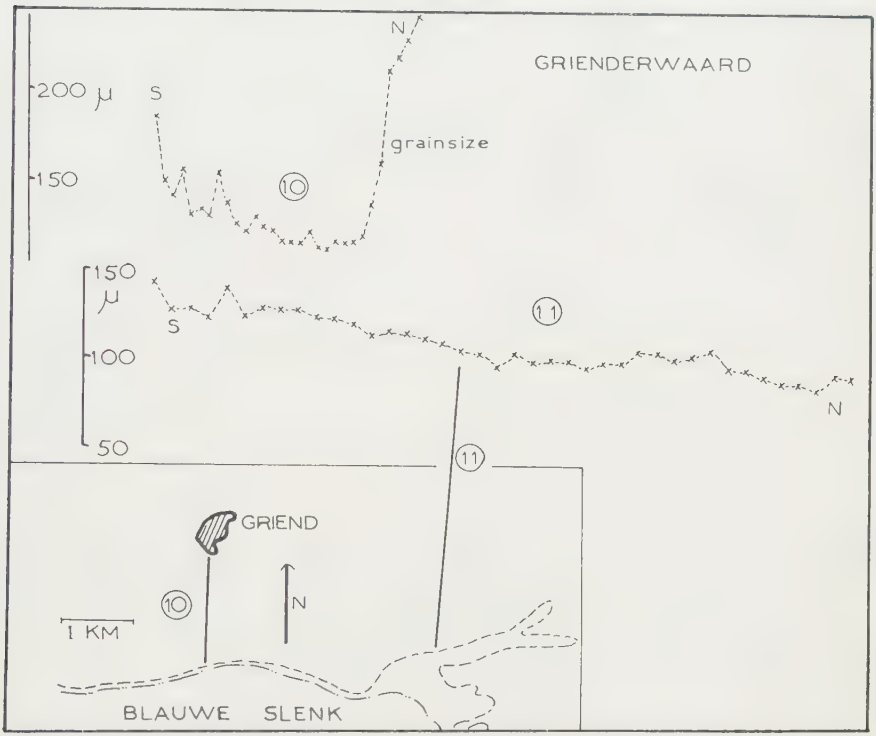


Fig. 10. Location and grainsize distribution of the traverses of tidal flats nrs. 10 and 11; compare fig. 1.

can be obtained. The fact, however, that, generally speaking, the explanation on the basis of wave influence most satisfactorily fits the observations makes up to a large extent for the lack of direct evidence. In figs. 8 and 9 (tidal flats 7 and 8), for example, the line representing median grainsizes runs approximately parallel to the line representing the depth profile, which may be explained by assuming wave influence to decrease here with increasing depth. If current velocity had been the decisive factor, a grainsize distribution precisely the reverse of the one observed would have been the result, since the highest part of the flat forms a watershed, where the currents reach minimum instead of maximum values.

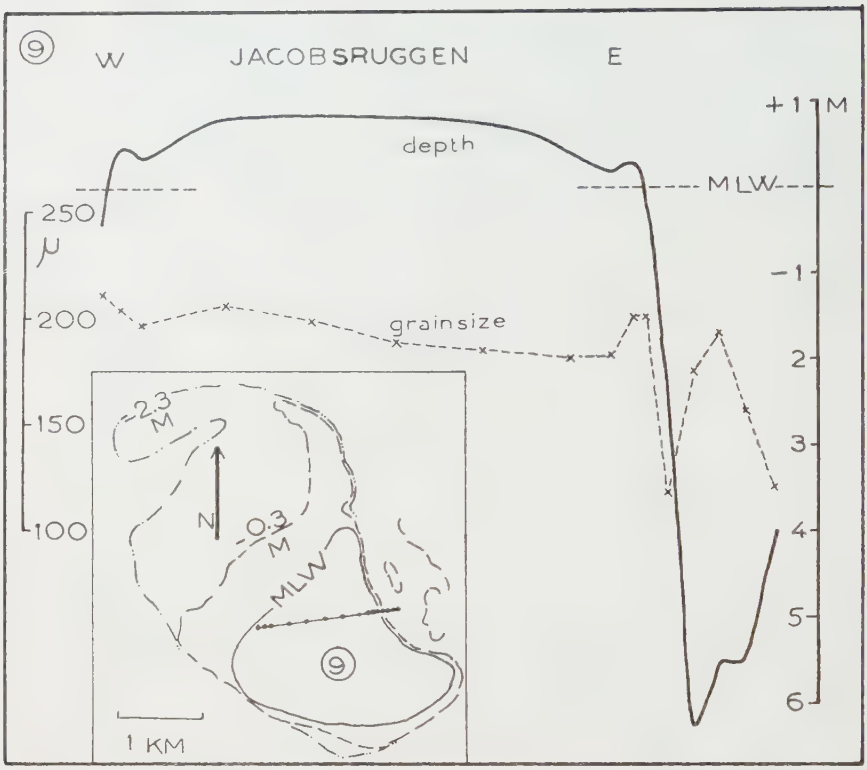


Fig. 11. Location, depth profile and grainsize distribution of the traverse of tidal flat nr. 9 and the adjoining Slenk channel; compare fig. 1.

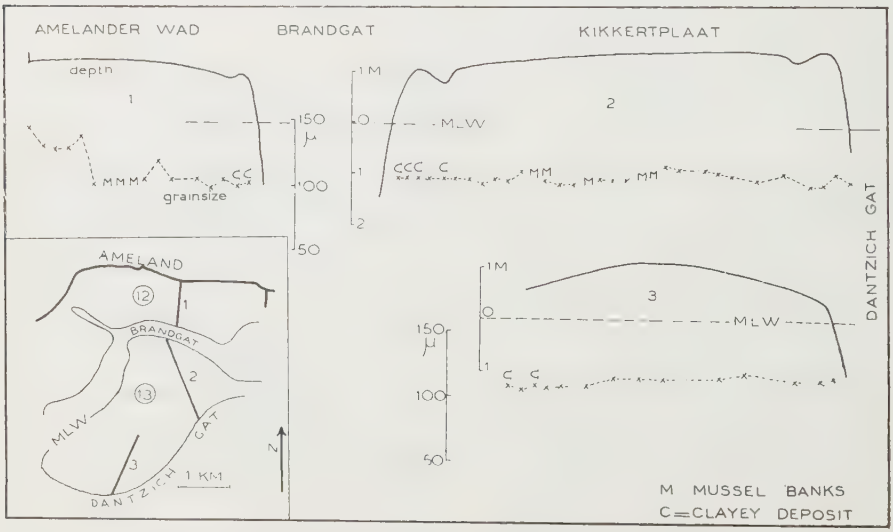


Fig. 12. Location, depth profiles and grainsize distribution of the traverses of tidal flats nrs. 12 and 13; compare fig. 1.

In fig. 10 (sections 10 and 11) a different type of distribution is encountered, the grainsizes decreasing regularly from South to North, although the tidal flat, according to the depth data from the sea charts, shows only very small differences in height (the coarse sands in the northern part of section 10 are located on the beach of the island of Griend and need not be considered here). The explanation of this grainsize distribution must be different from the one given above. High waves will be formed particularly in the deep and wide tidal channel "Blauwe Slenk", adjoining the flat in the South. When travelling a long distance in shallow water, the waves will gradually become weaker and in consequence the sediment will become finer with increasing

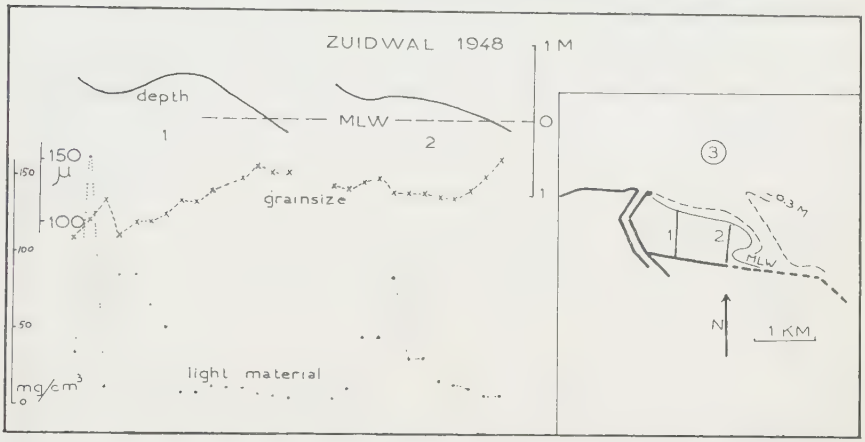


Fig. 13. Location, depth profiles, grainsize distribution and amount of light material of the traverses of tidal flat nr. 3; compare fig. 1. Light material expressed in mg of dry weight per cm³ of sediment.

distance from the channel. This effect may be especially influential on large tidal flats adjoining large channels.

Here also the question arises, whether current velocity cannot be responsible for the grainsize distribution observed. In the case of section 10 this assumption is difficult to refute, but neither is there any evidence to confirm it. In the other case the watershed is located halfway the section, the flood coming in from a channel North of the bank as well as from the "Blauwe Slenk". Hence the grainsize minimum ought again to be located halfway the section, if the currents were responsible, whereas it is actually found near the northern end. Therefore, an explanation based on differences in current velocity seems again improbable.

The above reasoning may also hold for the tidal flat nr. 9 of fig. 11, where median grainsizes decrease from West to East with increasing

distance to the Vlie tidal inlet. The same reasoning may further be applicable to the southern part of the tidal flat of fig. 12 (section nr. 13) adjoining the channel Dantzich Gat and for tidal flat nr. 3 near the Marsdiep (fig. 13).

Summarizing the above discussion we may conclude that the grainsize distribution on the flats will be principally determined by wave action. We saw already that in the channels current velocity is the most important factor. Therefore, there must be areas where the dominant influence passes from one factor to the other.

The sections of figs. 7, 8, and 11 are very elucidating in this respect. The smallest median grainsizes are found on the edge of channel and bank between 1 and 2 metres below the Mean Low Water line. We must assume that exactly here the combined action of tidal currents and wind is at a minimum; obviously the edge is out of range of the strong currents prevailing in the channel, but still too deep for strong wave influence. In the case of fig. 11 the grainsize minimum is comparatively high (120 μ), but in the other two cases the minimum is 75 and 90 μ respectively. Field descriptions and laboratory analysis show that these places are sufficiently calm for the deposition of such fine material as clay and organic matter together with the sand. An example of the distribution of light material along one of the sections is given in fig. 8.

In the cases described above the transition from predominant wave influence to the preponderance of currents could be sharply defined. Extensive areas will exist, however, where both currents and waves exert about equal forces. Such transition areas may for example be found on banks in the North Sea and on shoals in the Wadden Sea, which do not emerge at low tide. The proof that such areas are actually transition areas can only be furnished, however, after we have obtained a more exact knowledge of the actual forces exerted on the bottom by waves and currents than is available at present.

B. NORTH SEA AND MARSDIEP

Our knowledge of the distribution of median grainsizes in the North Sea and the Marsdiep (fig. 2) is based in the first place on a number of sections at right angles to the Dutch coast. The maximum distance of sampling stations from the coast is about 15 km and the maximum depth about 25 m. The chart gives only a schematic picture of grainsize distribution; more details are shown in fig. 3.

It appears to be possible to distinguish areas occupied by coarse deposits from those with fine sediments. Sands of median grainsizes above 200 μ occur in the tidal inlet and its principal entrances (Schul-

pengat, Westgat and Texelstroom; see fig. 1). Fine sands of grainsizes below 150 μ are deposited on the submarine shoal (Noorder- and Zuider Haaks; see fig. 1) in front of the tidal inlet. Further seaward the

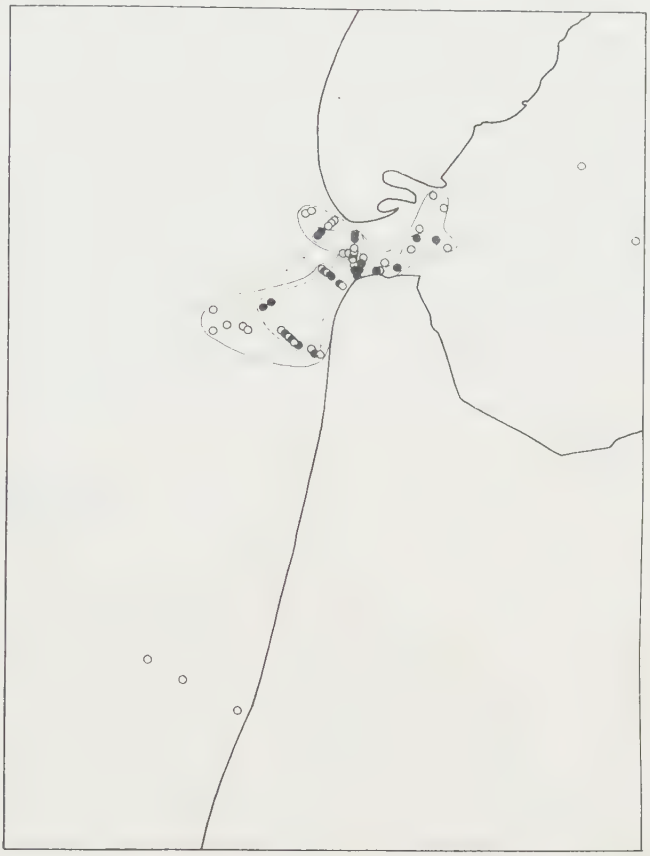


Fig. 14. Distribution of shells and shell remains in the Marsdiep area, based on field descriptions of all samples of figs. 2-5. Full circles: samples for the greater part consisting of shell material. Open circles: samples with many shells. No indication: samples with only few or no shell remains.

grainsizes again increase above 200 μ; North and South of the banks the 200 μ-isopleth bends towards the coast. Data from farther South published by DOEGLAS (1950) show that along the whole Dutch coast grainsizes are mainly above 200 μ.

The grainsize distribution of fig. 2 again suggests that just as in the Wadden Sea channels also in the adjoining part of the North Sea current velocity is the principal factor determining grainsize; if the power of waves were predominant, the coarses sandst ought to occur on the

shoals. This assumption can be confirmed for a large part of the area by current measurements of "Rijkswaterstaat" (FERGUSON, 1943) and our own observations on currents. Median grainsizes above 200 μ are found to occur in places where the average current velocity during calm weather exceeds a value of about 60 cm/sec (average along the vertical, and halfway spring and neap tide) or the maximum current velocity is higher than about 100 cm/sec. Within that part of the area where sands are finer than 150 μ, average current velocities are below 40 cm/sec and maximum velocities smaller than 70 cm/sec. From the area west of the Haaks, where the sediments are again coarser than 200 μ, no detailed current measurements are available; the average flood current velocity recorded by the lightvessel "Texel" amounts to about 60 cm/sec. All these values are only mentioned to give some idea of the correlation between current pattern and median grainsize distribution. Probably current velocities attained during storms can be much higher and are therefore far more significant for the grainsize distribution than those of calm periods. Moreover, wave action as a secondary factor may locally increase the power of the currents.

Before concluding the discussion on the grainsize distribution around the tidal inlet attention may be given to two related problems: the distribution of shell remains and the grainsize differences between tidal flats and beaches.

The coarsest sands in the Marsdiep (and probably in most other tidal inlets of the Wadden Sea) are mixed with very large amounts of dead shells and shell debris, mainly originating from the cockle (*Cardium edule*, see fig. 14). Since this animal inhabits in particular the tidal flats inside the Wadden Sea, most of the shells must have been transported to the tidal inlet from that direction. Transport of shells must therefore be possible at considerably lower current velocities than those occurring in the tidal inlet. Hence the shells must be transported very easily by the strong currents prevailing there. When they are nevertheless present in large quantities in the inlet and are not carried away from there, this must be due in the first place to the fact that the bottom slope favours transport in the direction of the deepest part of the area by force of gravity, so that the shells cannot escape from the tidal inlet, once they have arrived there. It must further be assumed that in the inlet shells are not easily buried below a sand layer. We will return to these points in detail in the next chapter.

Another interesting point to be mentioned concerns the grainsize difference between tidal flats in the strict sense of the word and beaches. If we compare the average grainsize values found on the beaches studied with those of neighbouring tidal flats, where the influence of environmental conditions is at first sight the same, the

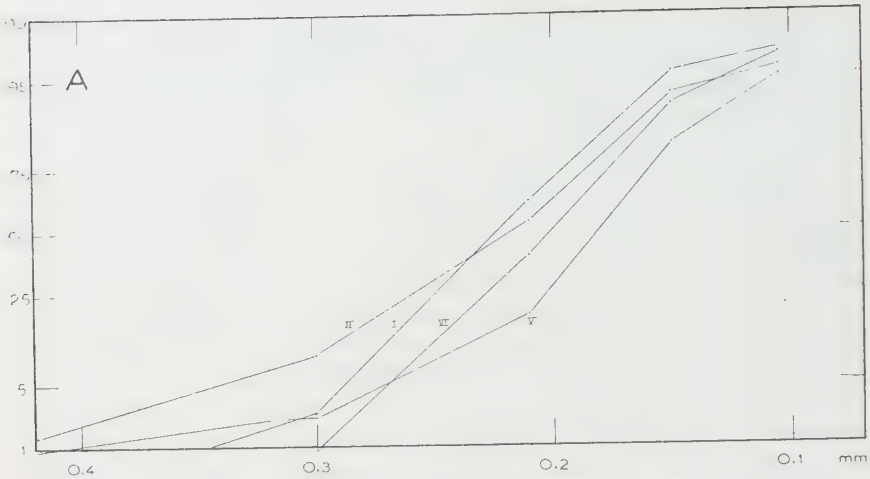
beaches appear to have considerably coarser deposits than the flats. This holds for the beaches of Texel and Den Helder (nrs. 2 and 5 of table 1) as compared with tidal flat nr. 1 and also for the beach of the island of Griend (upper part of section nr. 10 of fig. 10) as compared with the surrounding flat.

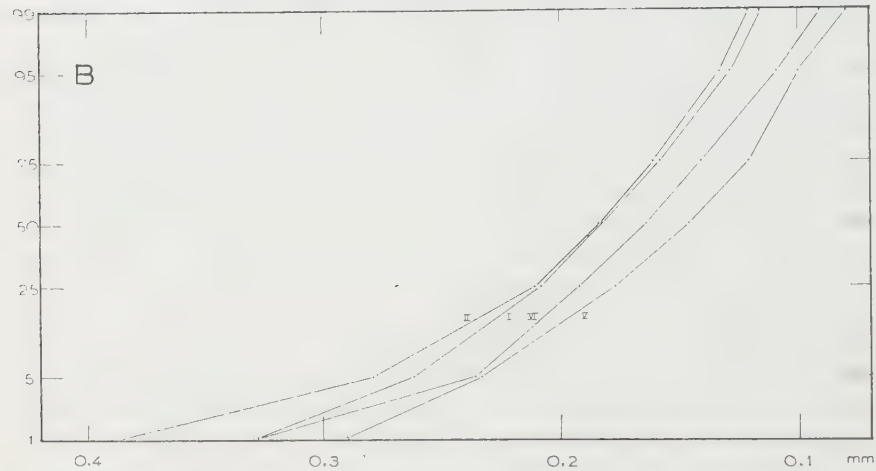
This grainsize difference must obviously be caused by the greater slope of the beach compared with that of the flat, which increases the power of the waves by causing them to break. In the surf zone, moreover, the water movement along the bottom towards the coast is swifter than in the opposite direction. Coarse material will only be carried by the strongest movement and therefore transported to the shore. Fine material will also be moved by the backwash and as the latter is aided by the slope (gravity) the finer material tends to be transported, seaward. Hence especially the coarse material is concentrated on the beach (INMAN, 1949). Although locally surf may also occur on tidal flats, especially on those directly exposed to the influence of the North Sea, its intensity will be considerably smaller than on the beaches.

If we now review the discussion of this chapter, our main conclusion must be that the grainsize distribution seems to reflect very faithfully the hydrodynamic conditions of the area studied, the intensity of water movement being the principal factor determining the degree of coarseness of the sands. This conclusion may indeed not seem very surprising, but is not self-evident in advance. In particular it would have been conceivable that the origin of the material and the direction of transport might have played a more important role.

V. GRAINSIZE FREQUENCY DISTRIBUTIONS

No attempt has been made in the foregoing pages to obtain an insight into the exact mechanism causing the grainsize differences. For that purpose it is necessary to consider, not only the median grainsize, but the complete grainsize composition of each sample. Stress must especially be laid on the significance of the size frequency distribution, since by means of the latter the problem of how sands are actually transported and sorted out in different environments, must be solved. KRUMBEIN (1936, 1938) has shown that the size frequency distribution of many sediments becomes more symmetrical when, instead of the diameter itself, the $-\log_2$ of the diameter in millimetres, indicated by Ø (phi), is given on the horizontal axis as the independent variable. The Ø-values 0, 1, 2, 3, 4, etc. correspond with the diametres 1, $^1/_2$, $^1/_4$, $^1/_8$, $^1/_{16}$ mm, etc. DOEGLAS and BREZESINSKA SMITHUYSEN (1946) introduced in sedimentology the plotting of cumulative curves on paper with a probability division on the vertical axis. On this paper normal cumulative frequency distributions are represented by straight lines. In this way





deviations of sediment composition from normal distributions can easily be studied.

A selection of samples represented in this manner is given in figs. 16-24. In this series of diagrams most Wadden Sea sands in fact yield lines which are approximately straight.

It must be observed here, that DOEGLAS and his collaborators actually preferred to represent their samples on probability paper with simply the diameter instead of its logarithm on the horizontal axis. They followed this procedure, since sediments of many environments of deposition, marine as well as fluviatile, generally show a smaller deviation from straight lines on arithmetic than on logarithmic probability paper. Moreover, they proved by means of a large amount of material that the method of representation followed leads to valuable interpretations concerning grain-size selection in different environments. This result seems to contradict our own

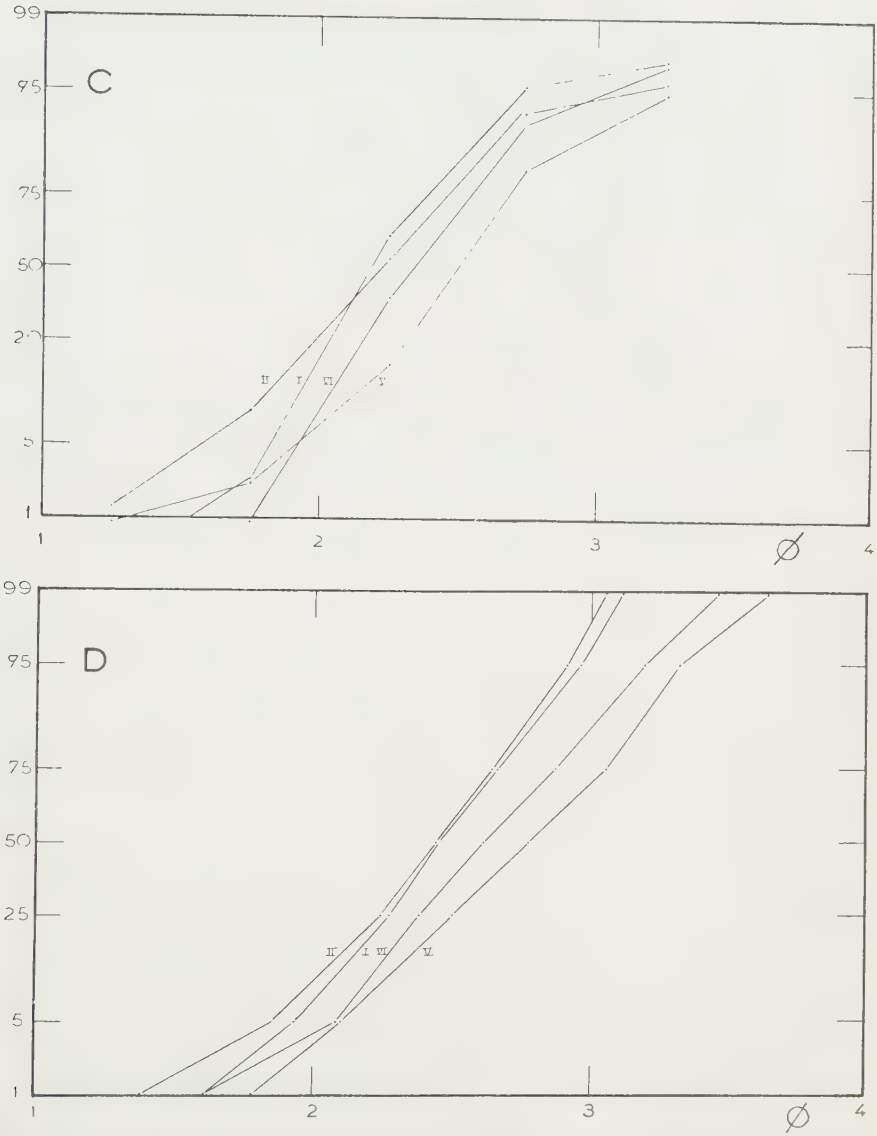


Fig. 15. Comparison between two different methods of analysis and two ways for graphic representation of grainsize frequency distributions; for explanation see text.

experience. However, many of the coarser sediments and also the coarse Wadden Sea sands studied by the above-mentioned investigators show, when represented on arithmetic probability paper, curves bending towards the left (viewed from the coarse part of the sediment towards the fine fraction; compare Wadden Sea sediment types I and II of Koning, 1950). These curves often become approximately straight on logarithmic probability paper. In these coarse sediments a very fine fraction is

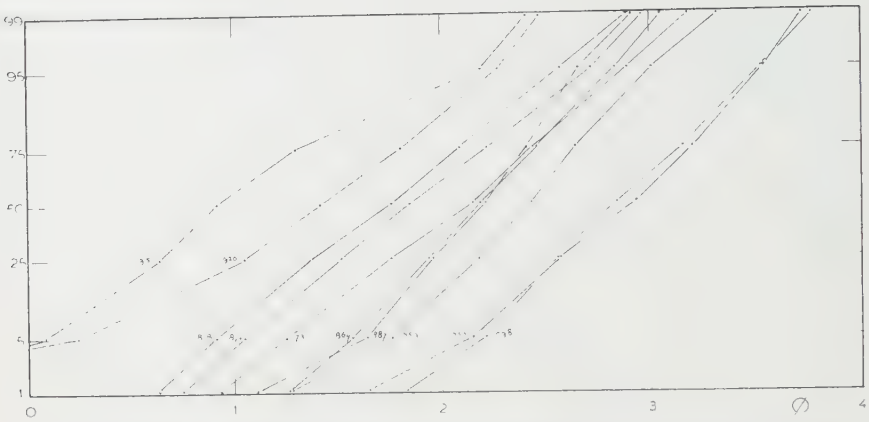


Fig. 16. Cumulative frequency diagrams of samples from the North Sea. For location of stations see fig. 3, small numbers; station 878 is also indicated in fig. 2.

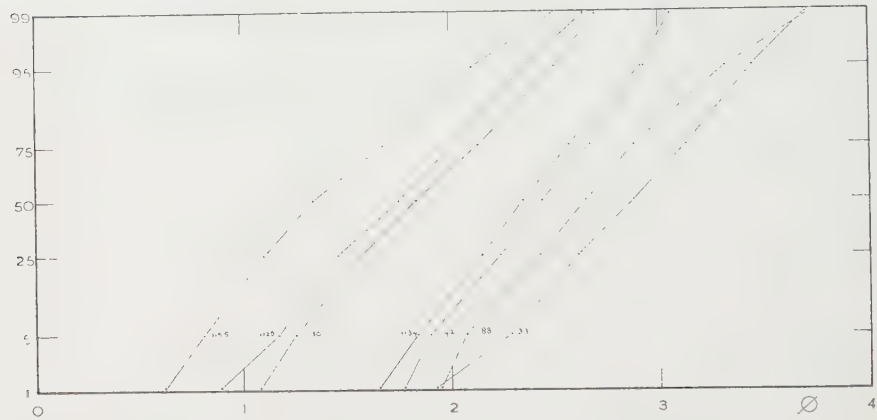


Fig. 17. Cumulative frequency diagrams of samples from the Marsdiep tidal inlet. For location of stations see figs. 2 and 4 (small numbers).

practically absent. As soon as the latter fraction becomes of importance, the analyses of the above-mentioned authors are no longer directly comparable with our own, since in the first case the fine material was disintegrated into elementary particles before the grainsize was determined, whereas in the second case the determination was carried out without such a preliminary treatment of the sample. The difference between the results of the two methods becomes clear from fig. 15 (A–D), in which four samples analysed both by Prof. DOEGLAS (A and C) and ourselves (B and D) have been represented both on logarithmic (C and D) and arithmetic (A and B) probability paper. For the analyses of DOEGLAS it is of little importance which type of paper has been used; for our own analyses plotting on a logarithmic scale evidently gives the best results. In the following paragraphs an attempt will be made to explain the formation of the sand frequency distributions on the basis of sand transport. In view of this purpose it seems better to consider the frequency distributions of samples in which the fine fraction has remained undisturbed.

When the series of lines of figs. 16 24 are more closely inspected it attracts attention that many of them run about parallel to one other, which means that in a logarithmic scale they show about the same degree of sorting (INMAN, 1952). This holds not only for sands from one tidal channel or shoal, but often also for those from different environments. Significant exceptions to this rule occur—especially beach sands and sands from the outer tidal flats are more thoroughly sorted than the other sands—but considered as a whole the material shows a

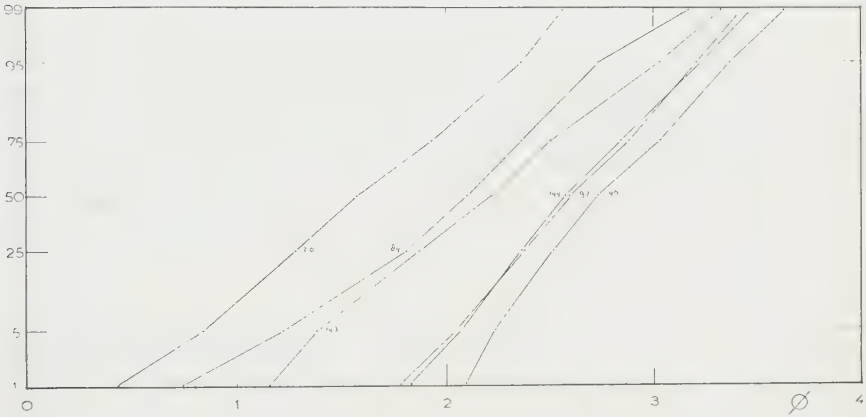


Fig. 18. Cumulative frequency diagrams of samples from the Vlie tidal inlet and adjoining Inschot channel. For location of stations see figs. 6 and 9 (small numbers).

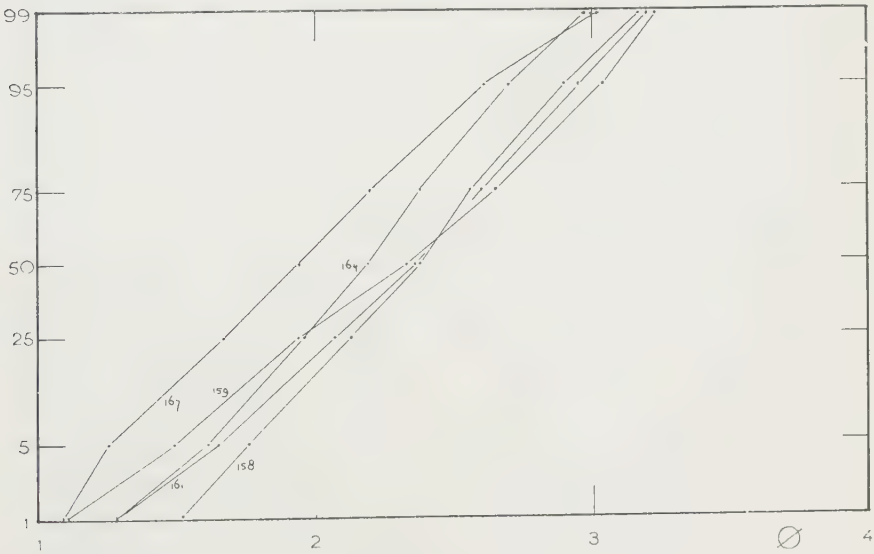


Fig. 19. Cumulative frequency diagrams of samples from the Dove Balg channel; see fig. 1.

remarkable uniformity. This uniformity becomes especially apparent if it is realized that a logarithmic diameter scale implies that one distribution can be derived from another by multiplying all diameters with the same factor. This indicates that for the process of grainsize selection only relative diameters are of importance; the frequency distributions may therefore be formed by competition between grains of different size. It also means that the factors determining the grainsize composition act in approximately the same way in different environments, bringing about only differences in coarseness of the deposits.

Starting from this point of view we may ask whether the mechanism of grainsize selection can be outlined more clearly. The simplest supposition would be, that from a certain initial frequency distribution the fine fraction is carried away, so that the coarse part remains behind. The fractions which are carried away, will be deposited in more quiet areas. The result would be that the coarseness of the distributions decreases with decreasing turbulence. On this basis a theory of sediment differentiation and mixing has been developed by DOEGLAS (1950). This theory can be summarized as follows.

Consider a bottom sediment showing a normal frequency distribution (and therefore represented by a straight line in the probability diagram) in equilibrium with certain current velocities. If the current strength increases, all sand grains below a certain diameter are winnowed out and will be carried away, leaving behind a sand assemblage, the distribution of which is no longer represented by a straight line, but by one curving towards the left (viewed from the coarse part of the sediment towards the fine fraction). If, on the other hand, the current velocity decreases, material below a certain grainsize may settle out. This would cause the curve to bend to the right. S-shaped curves may be formed in a somewhat more complicated way: suppose that the current carries away the fine part of a normal distribution and that from this fine part afterwards the coarser elements are deposited; the fraction remaining in suspension will then also show an S-form.—This reasoning holds in the case of an arithmetical division on the horizontal scale as well as in the case of a logarithmic division.

DOEGLAS and his collaborators proved that these theoretical distributions actually occur in nature. Their theory starts from normal frequency distributions, however, without giving an explanation for their formation. The selective action of the currents as described above seems to lead away from normal distributions instead of promoting them. To explain the differentiation into series of approximately straight lines such as predominantly occur in the part of the Wadden Sea studied, stress should be laid on the problem of how such uniform distributions are formed.

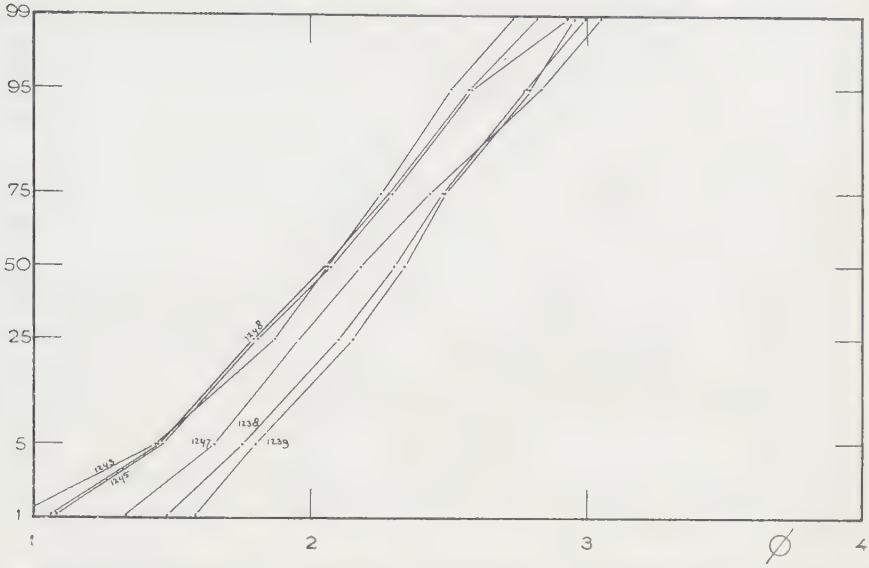


Fig. 20. Cumulative frequency diagrams of samples from the North Sea beach near Den Helder. Sampling locality nr. 2 of fig. 1. The samples were taken at distances of about 30 m along a traverse perpendicular to the low water line in the following sequence: 1238 (foot of dune), 1239, 1243, 1245, 1246 (low water line) and 1248 (below low water).

Although it is generally assumed that a residual transport of sand takes place from the North Sea to the Wadden Sea interior, this transport is of no importance as compared with the sand movement in alternating directions caused by the tidal streams. This movement exceeds many times the average transport in one direction, the amounts of sand carried back and forth being very large. The present author (Postma, 1954) estimated the average content of suspended sand in the areas studied at about 5 mg l during quiet weather conditions. At 1 m above the bottom values up to 500 mg/l were found in a few places during the period of maximum current velocity. Nearer to the bottom sand will be transported in still higher concentrations. Moreover, some observations during storms show, that in these circumstances the sand content often rises to much higher values; on one occasion a surface concentration of about 450 mg l was measured in a tidal channel of 12 m depth. But even the quiet weather values given above show that the upper layer of the sand bottom must constantly be in motion.

An important question is, whether grains of all diameters participate in this transport or whether only the light fraction is set in motion. Hjulström (1939, see also Bagnold, 1941, and Inman, 1952) has shown

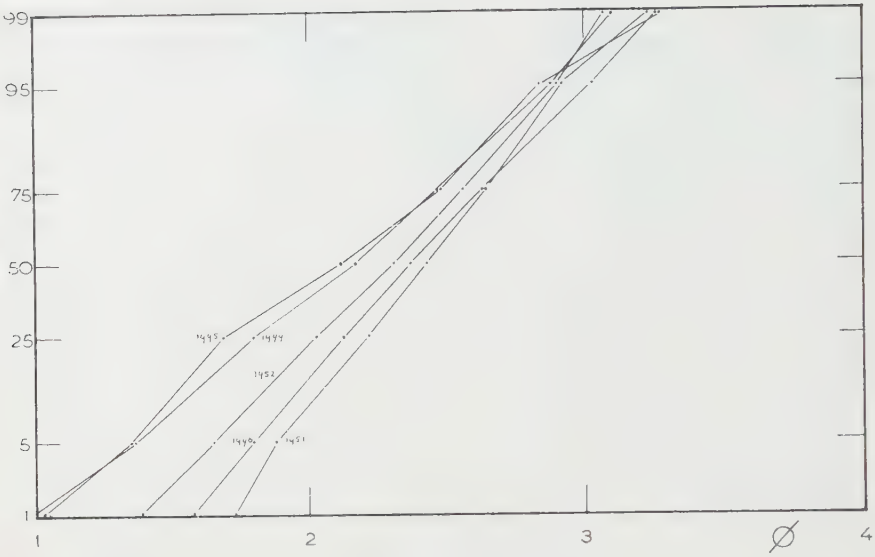


Fig. 21. Cumulative frequency diagrams of samples from the North Sea beach of Texel. Sampling locality nr. 5 of fig. 1. Position of samples: 1444 (low water line), 1445 (35 m shoreward), 1446 (70 m shoreward); 1451 and 1452 in shallow creek connecting the Slufter cove with the sea.

that sand grains of about 180 μ are easy to erode, whereas both coarser and finer sand is eroded more difficulty. For sand of 180 μ ($\emptyset = 2.47$) the minimum average velocity at which sand movement starts is 18 cm/sec (average along vertical). Sand grains of 1000 μ ($\emptyset = 0$) and 50 μ ($\emptyset = 4.32$) require velocities of only 25 cm/sec. These values hold for uniform material; in a mixed sediment both the finer and the coarser sand are probably eroded more easily.

In the area investigated current velocities even on shallow flats easily surpass values of 25 cm sec. Erosion of grains of all sizes therefore seems certainly possible, the more so as the influence of wave action must be added to that of the currents.

Once set in motion, transportation of sand is possible at much lower velocities than those mentioned above; the minimum transportation velocity for grains of 50 μ amounts to about 0.4 cm/sec; for grains of 180 μ and 1000 μ these values are 1.5 and 8 cm/sec respectively. Fine sand will therefore be transported more easily and over greater distances than coarse sand.

Much higher critical current velocities than those mentioned above have been published by Van Veen (1936), who measured sand transport in the tidal inlet between the Wadden Sea inlands of Vlieland and Terschelling. According to this author a current velocity of 100 cm/sec at 15 cm above the bottom is just sufficient to move sand of a deposit of an average grain diameter of 500 μ. It should be observed,

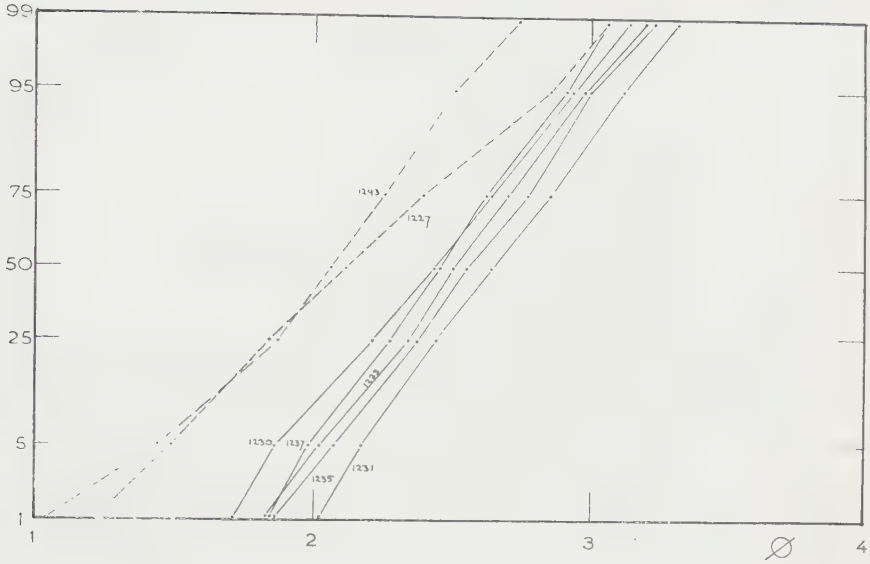


Fig. 22. Cumulative frequency diagrams of samples from tidal flat nr. 1 of fig. 1. Nrs. 1227 and 1243 represent samples from the Marsdiep (small numbers in fig. 2).

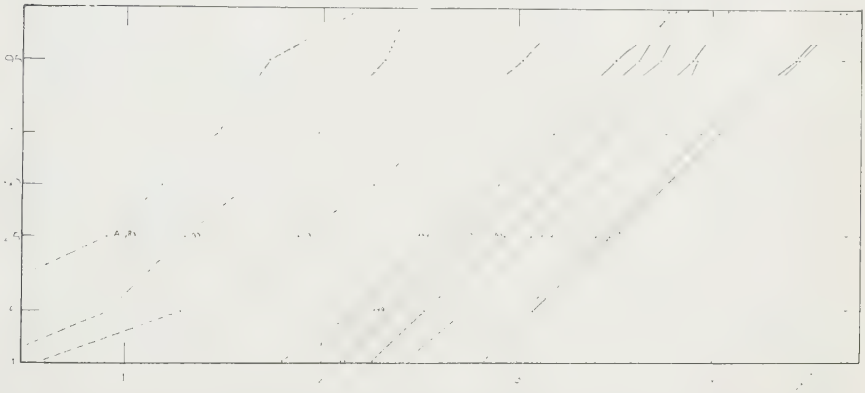


Fig. 23. Cumulative frequency diagrams of samples from tidal flat nr. 6 of fig. 1; compare fig. 7. The dashed lines represent samples from the adjoining Omdraai channel.

however, that VAN VEEN does not give the maximum grainsizes which are set in motion, as in sands with an average diameter of 500 μ grains of 1000 μ are still present in significant quantities. Besides, the above relation between sand transport and current strength applies to the moment at which the sand concentration at 15 cm above the bottom attains the comparatively high value of 2 $cm^3/10$ l or about 200 mg/l. The sand will already be transported, although in smaller concentrations, at lower current velocities.

As regards the distances over which the sand grains are transported,

there must be a great difference between the large channels and the tidal flats. In the Texelstroom channel, for example, ebb and flood carry the water back and forth over a distance of 20-25 km (POSTMA, 1954). Individual sand grains will not be moved so far, but it seems reasonable to assume that in one tidal phase they will cover distances of at least a few kilometres. For very sheltered intertidal flats LÜNEBURG (1956) has shown, on the contrary, that in the course of a tidal period sand grains are displaced over a distance of a few tenths of metres only. It should be added that small grains will in a short time be carried over much greater distances than large grains.

The present author determined maximum grainsizes of suspended matter in several water samples from different Wadden Sea stations, collected under normal weather conditions. Diameters up to 200–250 μ were found in all surface samples investigated, and up to 500 μ in all material from 1 m above the bottom. Sand grains of 1000 μ were found in most samples from near the bottom of the Marsdiep and the main tidal channels, including those of the Wadden Sea interior. It seems fairly safe, therefore, to state that all diameters up to 1000 μ can be transported in the channels, although this transport may be restricted to comparatively short periods around the maxima of ebb and flood current velocity. On the flats coarse sand may normally not be transported, but will certainly be moved during periods of strong winds. This means that practically everywhere in the area studied, the turbulence is from time to time strong enough to cause an intensive transport of sand grains of all diameters available.

The grainsize distribution at a given place cannot simply have been brought about, therefore, by the fact that small grains are winnowed out and carried away to more quiet places, whereas coarse material is left behind. Actually this process of selection is repeated infinitely in the direction of both the flood and the ebb current. As the material is carried back and forth within the same area, the sand grains are repeatedly submitted to the same set of selective forces and a complete adaptation of the areal distribution of grainsizes to the areal turbulence differences must have been attained. We will not enter into the question whether the average or maximum degree of turbulence, or yet another turbulence characteristic, is most important in this respect.

From the above point of view the formation of the frequency distributions can best be described by considering the areal distribution of grains of one diameter. In places where strong turbulent conditions prevail the relative amount in the sediment of these grains will be small, since they will remain in suspension and will be winnowed out more easily than larger grains. In very quiet places the relative amount of these grains will also be small, since they will reach these places with

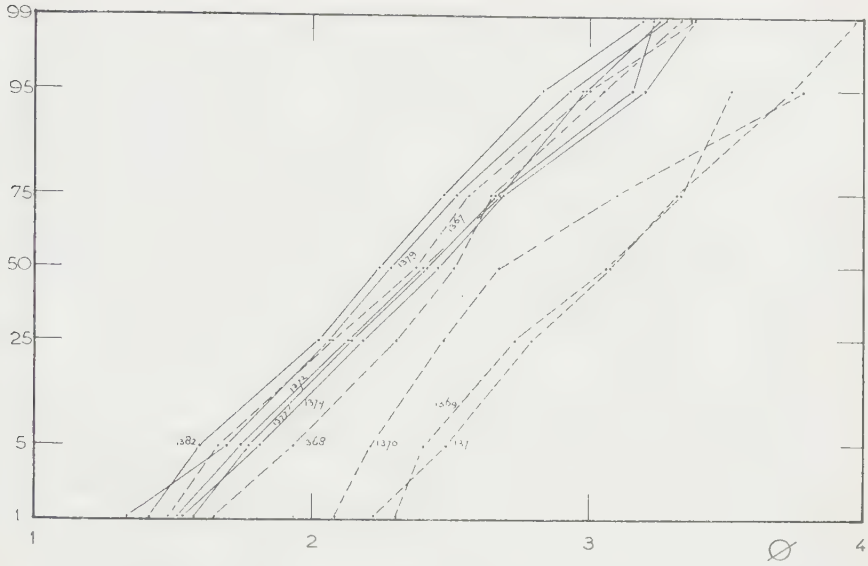


Fig. 24. Cumulative frequency diagrams of samples from tidal flat nr. 9 of fig. 1; compare fig. 11. The dashed lines represent samples from the adjoining Slenk channel.

more difficulty than smaller grains. As a result, the areal distribution of the grains considered will show a maximum of relative numerousness in a place with a special intermediate degree of turbulence. Different grainsizes will have maxima at different places. The areal dispersion of grains around the maxima, however, will not be the same for grains of different sizes: small grains, owing to their greater transportability, will be dispersed over a larger area and therefore show a less pronounced maximum than large grains.

The grainsize frequency distributions will reflect the areal distribution of the individual grains; they will therefore be unimodal and the grainsize spectrum will be broader when the sediment is coarser. Therefore, a close relation must exist between the median grainsize and the (arithmetic) degree of sorting of the frequency distributions. The composition of the actual frequency distributions, as shown by the measurements, is in agreement with this conclusion. However, the equal steepness of the frequency curves established on p. 341 even shows the existence of an exact proportionality between median grainsize and (arithmetic) degree of sorting. Moreover, the observations show that the frequency distributions are not only unimodal, but also symmetrical. An explanation of this proportionality and symmetry would involve a complete consideration of the laws of sand transport, which lies beyond the competency of the author. However, also without such final proof

the above theory of grainsize selection offers a reasonable explanation for the observed uniformity of the frequency distributions.

The main cause of this uniformity, as we saw above, is the existence of very turbulent conditions and currents in alternating directions. The formation of sediment assemblages in the way described by DOEGLAS *et al.* (see p. 342) occurs in environments where turbulence is too weak to cause transport of all grainsizes and where the sedimentary material is progressively transported in one direction. From this point of view both representations of grainsize selection do not contradict each-other, but they are operative under different circumstances.

VI. SUMMARY

The grainsize distribution of sands in the western Dutch Wadden Sea has been studied by means of samples analysed in the settling tube of VAN VEEN. Median grainsizes appear to increase with increasing current strength or increasing wave action. The selective action of currents is predominant in the tidal channels, that of waves on the tidal flats. Details of the distributions of median grainsizes are discussed.

The grainsize frequency distributions of individual sand assemblages can be represented by approximately straight lines on logarithmic probability paper. The uniformity of the sands is further reflected by the fact that all frequency curves have about the same steepness in the cumulative frequency diagram, independent of the locality of deposition. This uniformity is thought to result from repeated selection caused by continual tidal transport of sand grains of all sizes back and forth within the same very turbulent area.

VII. REFERENCES

BAGGERMAN, B., 1953: Spatfall and transport of *Cardium edule* L. Arch. Néerl. de Zool., **10**, 315–342.

BAGNOLD, R. A., 1941: The physics of blown sand and desert dunes. London, 265 pp.

DOEGLAS, D. J. and W. C. BREZESINSKA SMITHUYSEN, 1941: De interpretatie van de resultaten van korrelgrootte-analysen. Geologie en Mijnbouw, **8**, 273–296.

DOEGLAS, D. J., 1946: Interpretation of the results of mechanical analyses. J. Sed. Petrol.,**16**, 19–40.

DOEGLAS, D. J., 1950: De interpretatie van korrelgrootte-analysen. Verh. Kon. Ned. Geol. Mijnbouwk. Gen., **15**, 247–328.

FERGUSON, H., 1943: Verslag over de waarnemingen van het Rijksopnemingsvaartuig 'Oceaan' in het Zeegat van Texel; 1938–1939. Unpublished report Rijkswaterstaat, Den Haag.

HJULSTRÖM, F., 1939: Transportation of detritus by moving water. In TRASK, Recent marine sediments.

INMAN, D. L., 1949: Sorting of sediments in the light of fluid mechanics. J. Sed. Petrol., **19**, 51–70.

INMAN, D. L., 1952: Measures for describing the size distribution of sediments. J. Sed. Petrol., **22**, 125–145.

KONING, A., 1950: Observations concerning sedimentation in the Wadden Sea area, in the light of some granular analyses. Waddensymposium, Tijdschr. Ned. Aardrijksk. Genootschap, **67**, 82–88.

KREGER, D., 1940: On the ecology of *Cardium edule* L. Arch. Néerl. de Zool., **4**, 157–200.

KRISTENSEN, I., 1957: Differences in density and growth in a cockle population in the Dutch Wadden Sea. Arch. Néerl. de Zool., **12**, 350–452.

KRUMBEIN, W. C., 1936: Application of logarithmic moments to size frequency distributions of sediments. J. Sed. Petrol., **6**, 35–47.

KRUMBEIN, W. C., 1938: Size frequency distributions of sediments and the normal phi curve. J. Sed. Petrol., 18, 84–90.

KUENEN, D. J., 1942: On the distribution of mussels on the intertidal sand flats near Den Helder. Arch. Néerl. de Zool., **6**, 117–160.

LÜNEBURG, H., 1956: Untersuchung des Sedimenttransportes auf einigen Flächen des Wurster Wattes (Wesermündung). Veröff. Inst. Meeresf. Bremerhaven, **4**, 127-151.

MAAS GEESTERANUS, R. A., 1942: On the formation of banks by *Mytilus edulis* L. Arch. Néerl. de Zool., **6**, 283–326.

POSTMA, H. and J. VERWEY, 1950: Resultaten van hydrografisch onderzoek in de Waddenzee. Waddensymposium, Tijdschr. Kon. Nederl. Aardrijksk. Genootschap, **67**, 3–33.

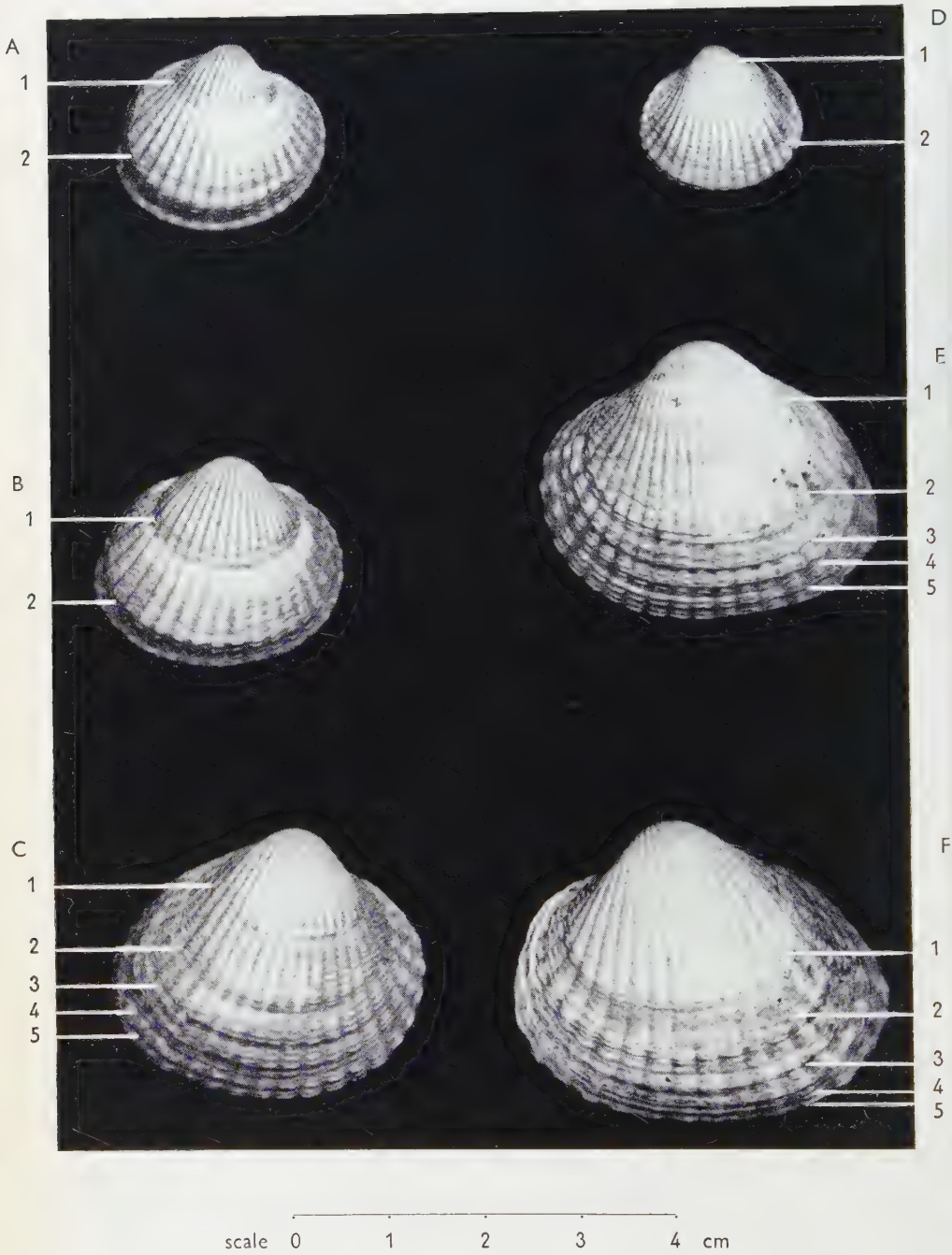
POSTMA, H., 1950: The distribution of temperature and salinity in the Wadden Sea. Waddensymposium, Tijdschr. Kon. Nederl. Aardrijksk. Genootsch., **67**, 34–42.

POSTMA, H., 1954: Hydrography of the Dutch Wadden Sea. Arch. Néerl. de Zool., **10**, 405–511.

VEEN, J. VAN, 1936: Onderzoekingen in de Hoofden. Thesis. Delft.

VERWEY, J., 1952: On the ecology of distribution of cockle and mussel in the dutch Wadden Sea, their role in sedimentation and the source of their food supply. Arch. Néerl. de Zool., **10**, 171–239.

Pl. I Landing on the Zeehondenplaat at sunrise. 10 Sept. 1948. (Photo Dr D. Kreger)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scale 0 1 2 3 4 cm

Pl. III A shallow creek inhabited by a very dense population of small-sized cockles; the stunted growth here may have been caused by the mussel-banks on the background. Staart van Schieringhals, Sept. 1948. (Photo Dr D. Kreger; Stereotype Koninklijk Nederlandsch Aardrijkskundig Genootschap).

← Pl. II Cockles from the Zeehondenplaat with two or five winter rings, indicated by white arrows. (Photo Mr Th. de Vries)

A. Flat-cockle with a small first, and a large second winter ring; cockle probably born in the creek and, after his first winter, transported to the higher part of the flat.

B. Flat-cockle with two winter rings.

C. Creek-cockle with five winter rings.

D. Creek-cockle with two winter rings, the first of which is very small ('secondary' spat).

E. Flat-cockle with abnormally small first winter ring; cockle probably born in the creek and, after his first winter, transported to the higher part of the flat.

F. Flat-cockle with five winter rings.

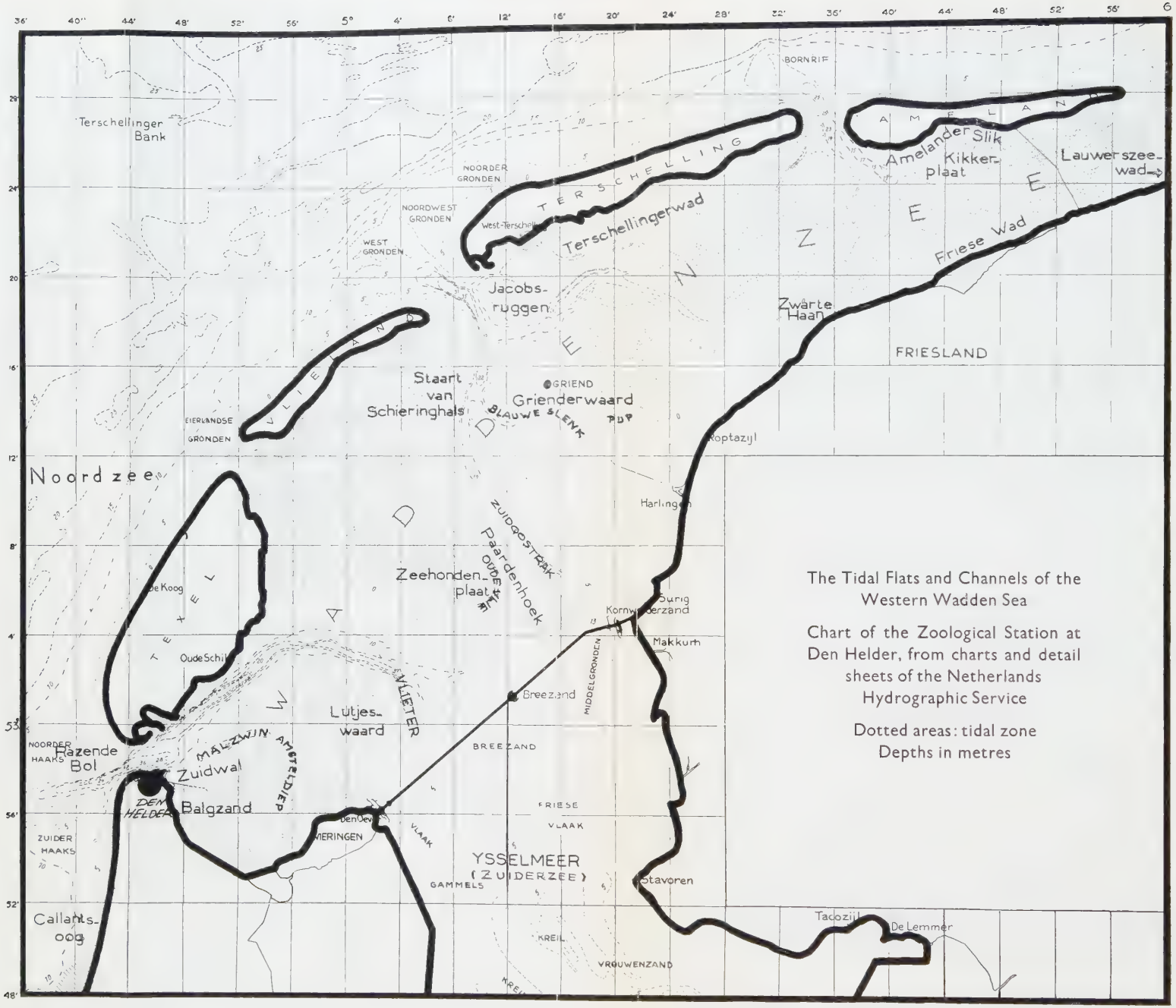

Fig. 1. The Tidal Flats and Channels of the Western Wadden Sea.

DIFFERENCES IN DENSITY AND GROWTH IN A COCKLE POPULATION IN THE DUTCH WADDEN SEA

by

INGVAR KRISTENSEN

(Zoological Station, Den Helder[1]*)*

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I. INTRODUCTION

Thanks to publications by THAMDRUP (1935), WOHLENBERG (1937), KREGER (1940), SMIDT (1951), and BAGGERMAN (1953), a good deal is already known concerning the ecology of the cockle (*Cardium edule* L.) in the Danish, German, and Dutch Wadden Sea. There remained, however, many questions of detail; of these, we are here more especially interested in the mollusc's population density and growth. In studying these factors, the chief difficulty is that one always has to do with populations consisting of different year-classes. Even in studying one and the same area for a number of successive years, so that one gets some insight into the course of development of the different year-classes, it is still quite difficult to take into account the direct and indirect influence of the different year-classes on one another. One would like to make a great experiment, demarcating test areas in different places in the Wadden Sea, and setting out, in each area, spat of one particular year only. This appeared to us impossible, but Nature managed it; viz. in 1947. During the winter of 1946/'47 the entire cockle population on the Dutch tidal flats was exterminated as a result of the exceptionally severe cold. We know for certain that, in the spring of 1947— at any rate in the shallows of the Western Wadden Sea — not even 1 ‰ of the normal cockle population had remained alive. On the top of the flats *all* cockles had disappeared. Notwithstanding this, the presence of a rich spat was ascertained in the summer of 1947. Evidently, a sufficient number of sexually mature cockles had remained in the surroundings of the shallows to make their repopulation possible. No doubt these older cockles had survived in deeper water—at any rate below the tidal zone. Although the cockle cannot maintain itself for long in the channels of the Wadden Sea, it is not unthinkable that large numbers of cockles are transported during the winter from the shallows to the channels, and there reproduce before dying. It is also

possible that the plentiful spat of 1947 partly derives from cockles living in the North Sea at some distance from the coast, because it has been proved that the cockle does succeed in maintaining itself below low-water level in the North Sea (vide p. 391).

It is nevertheless remarkable that, in 1947, when the population had been so considerably thinned out, the spat turned out so enormously rich.

Now it was found that the summer of 1947 was an extremely favourable breeding year also for other species. Thus, we found, among the sessile species, an abnormally rich spat of *Mytilus edulis*, *Pecten maximus*, *Pecten opercularis*, *Ostrea edulis*, *Cyprina islandica*, *Cardium crassum*, *Abra alba*, *Abra prismatica*, *Ensis siliqua*, *Polydora ciliata*, *Lanice conchilega*, *Balanus balanoides*, *Echinocardium cordatum*, *Ophiura texturata*, and *Astropecten irregularis;* but we also found that the spat of many non-sessile animals was exceptionally numerous; e.g. of *Crangon crangon*, *Nephrops norvegicus*, *Sepia officinalis*, *Engraulis encrasicholus*, *Solea solea*, and other (especially southern) species of fish.

The fact that 1947 was a favourable breeding year for so many species was doubtless due, first and foremost, to the high summer temperatures in that year. A warm summer appears to be favourable for successful breeding of many species of animals—not only for southern ones. The existence of a correlation between the quantity of successful spat and the temperature of the water during the spat's larval period was most clearly demonstrated with respect to *Ostrea edulis* in the Eastern Scheldt Estuary (Korringa 1940, p. 54), and with respect to *Engraulis encrasicholus* in the former Zuiderzee (Redeke 1907, p. 264). It seems that a warm summer is favourable also for the spat-fall of the cockle. In Holland, out of the years 1933–1939, and 1946–1954, the summers of 1934, 1936 and 1947 were the warmest; and it was precisely in these summers that the greatest quantity of cockle spat was produced. We find the same thing in Germany and Denmark; there too, 1934 was a rich spat year (Thamdrup 1935, Wohlenberg 1937). No data are known about 1936; but the very hot summer of 1947 again yielded—at any rate for the Danish tidal flats—a very rich brood of cockles (Smidt 1951).

Near Den Helder 1929 and 1939 were also rich spat-years, although neither of the two summers was particularly warm. In both years, there had been periods of severe cold during the preceding winters, which is catastrophic for the cockles in the tidal zone. In Denmark too, the quantities of spat were remarkably great after an ice-winter, e.g. in 1928 and 1929 (Thamdrup 1935), and in 1942 and 1947 (Smidt 1951). It appears improbable that, after a cold winter, the production of eggs should be greater than normal. On the contrary, owing to the mortality

among such large numbers of adult cockles, the total production of eggs after a severe winter must surely be quite small. Evidently, however, the mortality of the larvae in such years is exceptionally low, so that the quantity of spat that reaches the sedentary stage is large. The fact that, in such years, the mortality of the larvae is so low may be partly due to the smaller number of plankton-eating animals in a shallow area such as the tidal-flats, after a cold winter. Our observations show that, after the ice-winter of 1946-'47, the number of Lamellibranchiata and Cirripedia was very small during the larval period of the cockle (May), while there are indications that the number of plankton-eating Polychaeta and fish were also small at that time.

Furthermore, the cockle larvae were able, in 1947, to settle on a practically depopulated bottom, without the competition of older cockles, mussels, etc.

It is probable, therefore, that the combination of an ice-winter and a warm summer following it constituted the conditions for the rich cockle spat in 1947.

In most years, the spat-fall of the cockle takes place chiefly in the beginning of June; but there may also fall a rather considerable quantity of spat later in the summer—especially in August. In 1947, however, no such "secondary" spat was found. Possibly the hibernating adult cockles had meanwhile died (see above); but the chances of any secondary spat were already rendered nugatory in advance by the invasion of large numbers of plankton-eating animals in the course of the summer. For our own investigation it was a most favourable circumstance that, precisely in 1947, the entire spat-fall took place in June, with the result that the population was exceptionally homogeneous as regards age.

After 1947 there followed a series of years with a very poor spat-fall, so that the 1947 cockle class was completely predominant for many years, and even remained, in some places, the quantitatively most important year-class until as late as 1953. This provided the investigation with a precious chance: it was possible to study one and the same year-class for a series of successive years. The present publication refers to only part of the investigation, and endeavours to answer the question, *how a given year-class of cockles develops in the course of the years, under different habitat-conditions, considering both the population density and the animals' growth.*

In order to get some insight into the density and growth of the cockle in different parts of the tidal flats of the Western Dutch Wadden Sea (fig. 1), samples were collected in various places, both on the flats and in the channels. Our initial intention was to take samples at the same points for a series of successive years. It was found, however, that not

all places from which samples had been taken provided comparable data; in some cases because the constitution of the flat had been radically changed as a result of hydrographic causes, and in other cases because a quantity of cockles had been fished away from the locality for consumption purposes. In the end there was only one area left that continued to meet our demands practically entirely, and on which we accordingly concentrated the greater part of our investigation; this was a flat situated near the Oude Vlie, and which we called "Zeehondenplaat", because of the frequent occurrence of "Zeehonden" (= seals).

But it was found that the results obtained on the "Zeehondenplaat" did not always correspond to those on the other flats. This was largely a result of the considerable differences in profile of the flats between high and low water level. In our investigation we found three different types of profile, which have been sketched in fig. 2.

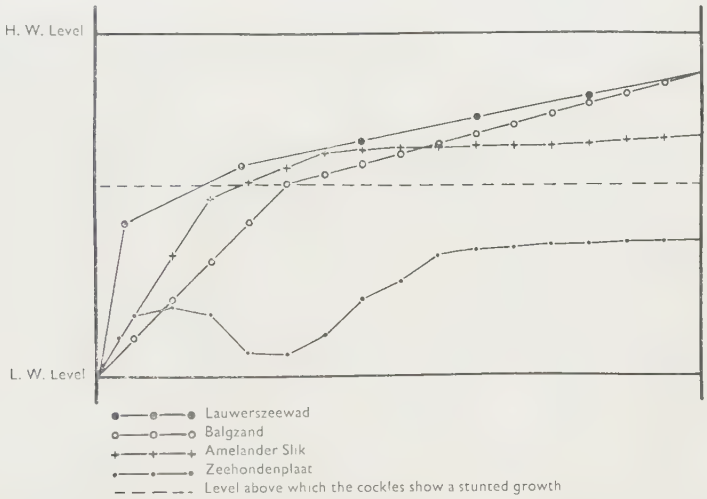

Fig. 2. Profile of the Tidal Flats between Low- and High-water Level.

The first type of profile we distinguished is the Zeehondenplaat-type, i.e. the type of a tidal flat lying low over its entire length, whose highest parts fall dry for only 4 hours maximally per tide. Such flats are usually surrounded by deeper water, and have the character of a sandy shoal rather than of a mud-flat. In some cases there may be at the low-water line, along a deep channel, a relatively high sand-ridge, behind which there is a tiny, shallow creek. This type of flat includes the Zeehondenplaat, the Paardenhoek, the Staart van Schieringhals and the Jacobsruggen.

The second type we found was the Balgzand-type, in which the flat rises, at first somewhat steeply, and later with a smaller gradient, from the low-water to the high-water level. Here, too, there may be a high ridge along the low-water level. On the high-water level the flat often passes into a salt-marsh. In most cases the entire structure leans against the foot of a dyke. This type of mud-flat is found at the Balgzand, on the south-west side of the Zuidwal, on the Terschelling Flat and, locally, along the Frisian coast. A variant of this type occurs in the Lauwerszee, where the flat has a very steep bank at the low-water line, with the result that it falls dry for a long time over practically its entire length. This is also the type of tidal flat profile which, it appears, occurs frequently in Denmark (Thamdrup 1935, Smidt 1951).

The third type to be mentioned is that of the Amelander Slik; here, the flat rises fairly steeply from the low-water line, after which it continues practically horizontally over a large expanse. We found this type also at the Kikkerplaat, and near Griend.

This classification into types of tidal flat does not pretend in any way to be a contribution to the relevant geomorphology. It merely purports to lay stress on the differences in profile shown by tidal flats; differences which proved of importance in investigating the ecology of the cockle.

The population density and growth of the cockle will be discussed in in separate chapters. In this, both the abiotic and the biotic factors which may be of influence, will be dealt with. Several of these factors will be discussed repeatedly, because they may influence the settlement of the spat, the displacement of the older cockles, the mortality, and the rate of growth. We preferred this to the alternative of considering all influences of one single factor together. The reason why we did not follow this last method is because we wish to give to the problem of the regulation of density and growth of a cockle population the central place it deserves. Neither is the order of sequence in which the different environmental factors are dealt with, primarily determined by any classification of environment. It is determined by the manner in which, in our view, the biological problem can best be studied.

During this investigation, the personnel of the Zoological Station at Den Helder, and a large number of students, have kindly given their assistance in all sorts of ways. I especially owe a debt of gratitude to Mr. A. D. G. Dral, for his assistance in the elaboration of the data; to Mr. J. W. de Blok, Dr. L. F. Kamps, Prof. Dr. C. J. van der Klaauw, Prof. Dr. D. J. Kuenen, Dr. H. Postma, Dr. J. Verwey, Dr. M. G. Vroom, and Dr. J. Westenberg for their criticism and advice on the manuscript; and to Mr. E. van Loo for the translation.

II. TECHNIQUE

The method of investigation consisted in the repeated determination of the density and size of the cockles, along certain traverses whose position was determined with the aid of a sextant or compass.

On the Zeehondenplaat, the starting point and the direction of the traverse were indicated, from 1949 onwards, by means of iron rods stuck into the flat, to ensure that the samples of succeeding years should be satisfactorily comparable (fig. 3).

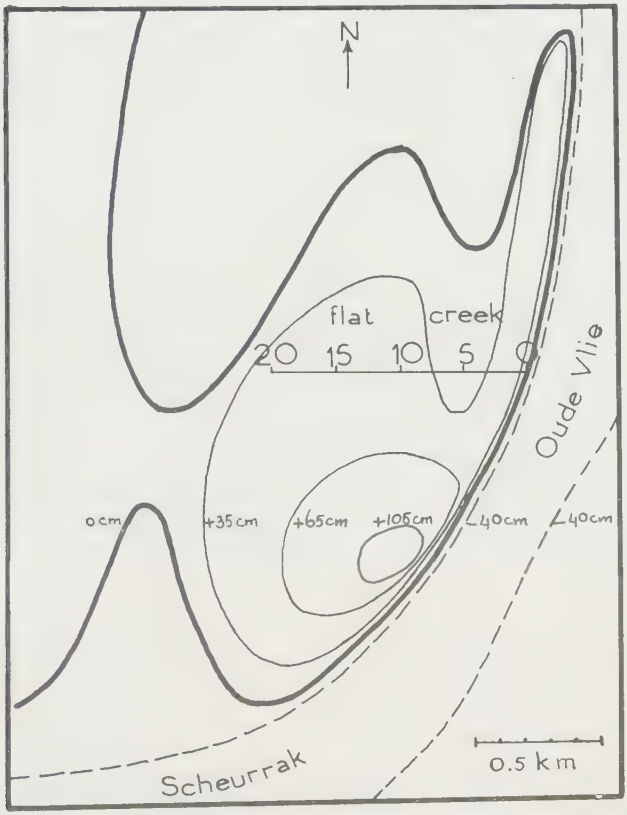

Fig. 3. Chart of the Zeehondenplaat (from Baggerman, 1953, modified).

As many topographical, sedimentological and biological data as possible were collected from each sampling point separately. On the flats that had emerged, the differences in the water-level were determined between sampling points located at equal distances from each other.

In the channels, the collecting of samples was done from a boat (cutter), with the aid of the Van Veen grab, which covers an area of

20 dm^2. On the flats, the samples were collected by excavating the bottom over 20 dm^2 to a depth of about 3 cm.

The size of the sand-grains was afterwards determined in the laboratory by means of the Van Veen settling tube (VAN VEEN 1936, p. 165–167), and the median grain sizes were calculated (cf. POSTMA 1957).

The live cockles of each sampling area were collected from the sample on the spot. In the case of very small individuals, the sample was sifted over a sieve of 1 mm mesh-width, the remains in the sieve being afterwards sorted out in the laboratory. Our method of sifting was inadequate for the quantitative collection of animals smaller than 1.5 mm. For this, we should have needed a finer sieve; but such a sieve holds back so much sand that sorting out the tiny cockles becomes extremely difficult (cf. BAGGERMAN 1953, p. 318). We did occasionally find animals with shells shorter than 1 mm, but only cockles longer than 2 mm were collected quantitatively.

Where, in what follows, the term "spat" is used, it refers to the small cockles in their first summer, from the time they have reached a size of 1–2 mm.

The collected cockles were counted, and measured with one millimetre accuracy. As measure of length, the greatest length was taken, i.e. from the anterior to the posterior side of the shell. In addition, of each cockle older than one year, one or more winter-rings were measured. Further—in some cases—the height of the shell (from dorsal to ventral), and the greatest thickness of the cockle (from the left-hand to the right-hand shell) were determined.

In a few cases the dry weight, both of the soft tissues and of the shell, was determined. Finally, some counts were made of the number of diverging ridges on the shell, and the number of ripe eggs in the ovary.

III. LOCAL DIFFERENCES IN DENSITY

Generally speaking, the density of a cockle population consisting of more than one year-class, is determined by the intensity of the spat-fall in the different years, by the displacement after the first settlement, and by the mortality among the different year-classes. In our investigation, only one year-class of cockles was chiefly involved. Since no comparison with other year-classes could be made, nothing much can be said about the population density as compared to other years. We studied the local differences in density that can be investigated precisely in one and the same year-class.

We discuss below the various factors responsible for the density and the differences in density between one area and another.

A. SPAT-FALL AND FINAL SETTLEMENT

During recent years it has been demonstrated, with respect to many sedentary species, that the larvae do not merely let themselves passively sink to the bottom, but endeavour actively to find a suitable substratum (cf. THORSON 1950, and WILSON 1952). But the investigations made up to the present chiefly concern those species of animals which are found on only one type of substratum. Little is known about any possible preference on the part of the larvae of species—such as the cockle—which are found on different kinds of substrata. An exception is the fairly euryokous species *Venerupis pullastra*, whose larvae, according to QUAYLE (1952), are so little fastidious in the choice of a substratum that they also settle down in places where the bottom offers less favourable chances of survival.

Although we have not, in the case of the cockle, any direct observations at our disposal concerning the falling of the spat, one may conclude from the presence of cockle-spat on a substratum varying between muddy sand (median grain size 50 μ) to coarse sand (176 μ), that the larvae do not show a great deal of preference either, when choosing a place of settlement. It is only in soft mud that no cockle spat is found, at any rate no spat larger than 500 μ. It is possible that this type of substratum is avoided by the cockle larvae when settling down.

TABLE I

Distribution of the cockle-spat on the Zeehondenplaat on July 2nd, 1951, and July 13, 1955

point	1	2	3	4	5	6	7	8	9	10	11	12
1951												
number of cockles of 1 mm	10	23	30	46	111	19	16	8	12	17	20	31
2 mm	32	21	66	85	203	60	20	6	3	9	5	16
1955												
number of cockles of 1 mm	61		72		133			30		26		29
2 mm	22		23		289			10		4		3

The observations to be discussed in the following served to obtain a clearer insight into the larvae's manner of settlement. We shall first examine the situation on the flats. On the Zeehondenplaat—the one most closely investigated—it appears that, every year, spat of an average size of about 1 mm is distributed more homogeneously over the flat than the slightly larger cockles of 2 mm. This phenomenon is especially

manifest in years yielding a numerous brood, as in 1951 and 1955 (see table I). (We have no observations, concerning these small animals, with respect to the rich spat-year 1947.)

From these figures it may be deduced that the local differences in density of the slightly larger spat are not primarily caused by the spat-fall, but that they arose—at any rate to a large extent—at a later period. It would be interesting to know how this shift came about and we shall now go into this more closely.

1. *Influence of the current on the settlement of the spat*

Linking up with our investigation, Miss BAGGERMAN (1953) studied the question of the extent to which these local differences in density might be caused by the young cockles being transported by the current. Unfortunately she was unable to include cockles smaller than 600 μ in her investigation, because these tiny cockles were concealed among the grains of sand. But it was found, with respect to spat above 600 μ, that final settlement—at any rate on the Zeehondenplaat—does not take place until the animals have reached a size of 1800–2000 μ. After this, as is clear from our own investigation (see p. 372), no further essential changes occur in the distribution. The reason for this is probably that the cockles have then become too heavy to be washed away. The distribution effected until that moment is the result of passive transport. The largest numbers of cockles, therefore, will be found in places where the bottom is least exposed to the influence of the current; a finding which is also clearly demonstrated by Miss BAGGERMAN's investigation.

Where, on the other hand, the bottom of the tidal flat—as a result of the action of the wind, surf and current—consists of clean, coarse sand, the numbers of cockles will be relatively small.

On flats which—like the Zeehondenplaat—are surrounded on different sides by deeper water, the largest numbers of cockles are found on the leeward side, i.e. on the eastward slope of the bank (Jacobsruggen, Kop van de Staart van Schieringhals, Paardenhoek; Groningen; see table II).

The numbers of cockles are especially large in sheltered places, as stated above; e.g. in shallow creeks (Zeehondenplaat, Grienderwaard, Balgzand); in areas where the tubes of the pipe-worm *Lanice conchilega* protrude above the surface of the bottom (Zuidwal), and even behind artificial screens composed of twigs and branches (Uithuizer Flat, Groningen; observation by Dr. J. VERWEY).

As another illustration of the part played by the sheltered condition of the locality, I will mention the following observation, made on a flat with a dense *Lanice*-population.

TABLE II

Maximum number of cockles found per 0.2 m².

flat	date	numbers of specimens on westward slope	numbers of specimens on eastward slope
Jacobsruggen	4 Sept. 1953	4	139
Kop van de Staart van Schieringhals	7 Sept. 1948	6	79
	25 July 1949	36	111
Paardenhoek	4 Sept. 1947	16	141
	8–9 Sept. 1948	20	187
	7 July 1949	47	128

On June 23, 1948, the density of the *Cardium* spat larger than 1 mm in length, both within and outside the *Lanice*-field on the Zuidwal, was, on a rough count, ca. 2500 specimens per m². The largest animals measured 1.5 mm, but the majority consisted of animals of about 1 mm. Since—as we mentioned before—we worked with a 1 mm sieve, it is also possible that the majority were smaller than 1 mm. On July 7 of the same year the density within the *Lanice*-field amounted to ca. 5000 specimens per m². The size of the animals sifted from there was fairly regularly divided between 1 and 2.5 mm. Outside the *Lanice*-field the density was the same as on June 23rd, while the cockles still had the same small size, i. e. < 1.5 mm.

This means that, in the *Lanice*-field, the spat of June 23rd had had an opportunity to grow up, and, at the same time, had been supplemented by newly arriving spat. The spat outside the *Lanice*-field, on the contrary, had been washed away by the current, and been supplanted by fresh spat of 1 mm.

It is clear, therefore, that the density of cockles in sheltered places is due to the action of the current, which easily transports the youngest animals from less sheltered places, whence they automatically land in sheltered places, where the bottom is more stable.

It follows from these observations that the spat along a surf-beaten coast, or a bank exposed to the prevailing winds, will have little chance of permanent settlement. And in fact, only very little cockle spat is found on the beaches of the open coast of Holland, apart from a few individuals that succeed in maintaining themselves in slightly sheltered creeks along the shore. We have never found a single cockle on a shoal outside the tidal inlet, emerging at low tide, such as the "Razende Bol".

One might further expect to find, in the channels of the Wadden Sea at sheltered places, a dense cockle population, because large quantities of spat should come to rest there after being washed away from the

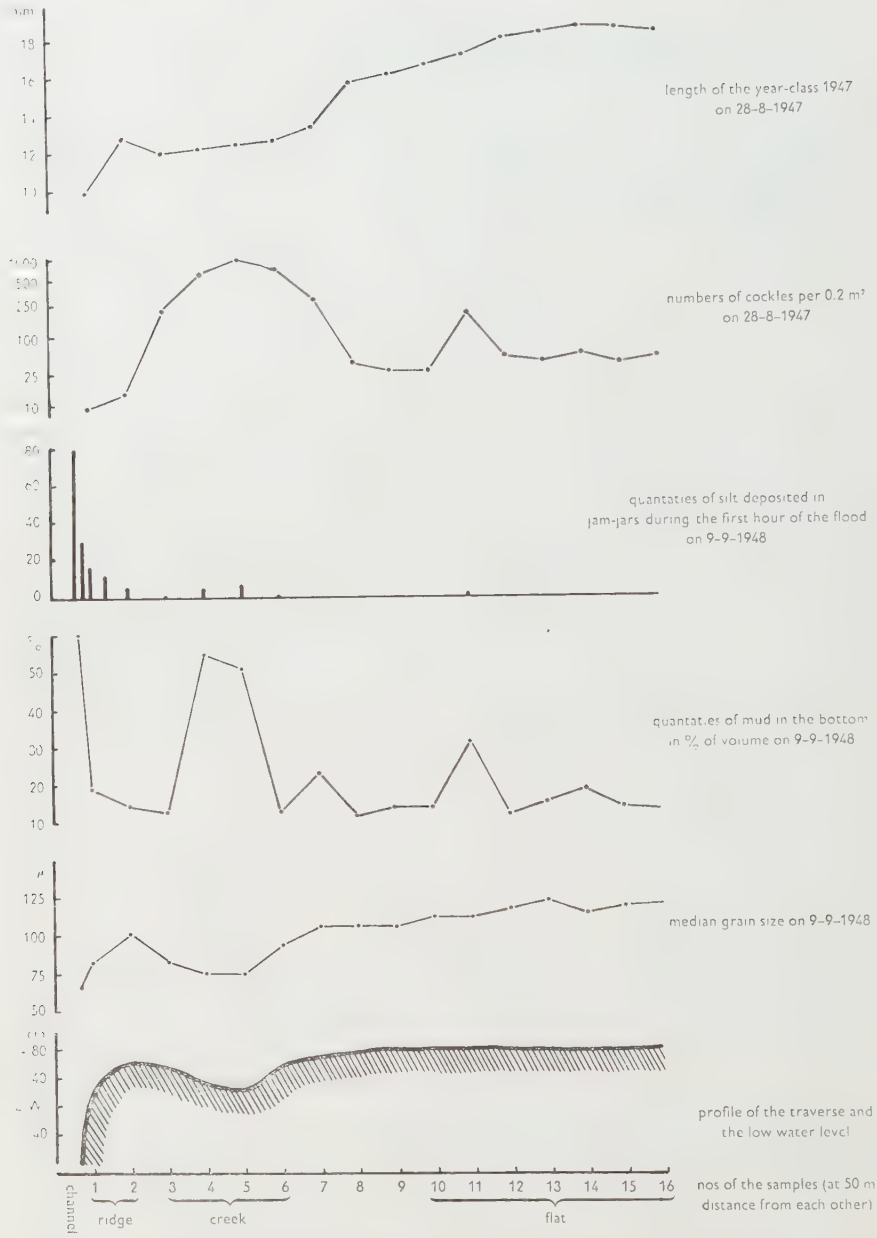

Fig. 4. Data from the Zeehondenplaat, relating to the median grain size; quantities of mud in the bottom; quantities of silt (incl. detritus) collected in jars dug into the flat; numbers of cockles, and length of the cockles. The numbers 1–16 are the numbers of the sampling areas on the traverse from east to west.

banks. Remarkably enough, however, only now and then did we find any cockle spat below a depth of 2 metres in more than 1000 bottom samples and we rarely found any larger cockles. It seems that the conditions of life in deeper water are too unfavourable for the cockle in the deeper parts of the Wadden Sea—a circumstance to which we shall revert further down.

Now that we know that—at any rate on the flats—the density of the spat depends to a considerable degree on the wind and the strength of the current locally, one might expect that there would be a close correlation between the structure of the bottom and the cockle density, since the structure of the bottom of a tidal flat, too, is chiefly determined by both wind and current. But KREGER's investigation (1940) demonstrates that no such correlation can be found when we compare one flat, as such, with another. We cannot give an explanation for this remarkable fact. According to KREGER, there may admittedly be, on the separate flats, a correlation between the density of the cockle spat and the "flocky material" present in the bottom, and an occasional correlation between the density of the spat and the grain size of the sand. This was also confirmed by our own investigation. Thus, on the Zeehondenplaat we did find a certain correlation between the density of the cockle spat and the quantity of mud in the bottom (fig. 4.) It is only along the channel, at the low-water line, where the bottom is very rich in mud, that no cockles are found. But as we already remarked above, the large channels in the shallows in the Wadden Sea appear to be unsatisfactory as a habitat for the cockle. There further appears to exist a correlation, on the Zeehondenplaat, between the density of the cockle population and the grain size of the sand (fig. 4); the finer the sand, the greater the density. That this correlation is not an accidental one in this case, is clear from the fact that the distribution of the spat over the flat is practically equal each year (fig. 5), while the distribution of the grain sizes, too, did not change very much during those years (fig. 6). The correlation referred to here is surely bound up with the typical bottom profile of this flat. The small, shallow creek has fine sand and a lot of cockles. The fact that here the bottom consists *everywhere* of fine, muddy sand indicates that the quantities of water passing through this creek are not sufficient to keep it at a certain depth. This implies that the local situation, viewed from a hydrographic and sedimentological angle, was not in a state of equilibrium. And in fact, we see how, during subsequent years, this creek is gradually filled up with sand. At first (in 1947), therefore, there existed a sheltered condition which was "abnormal" with respect to the current passing through the creek in question. As the creek eventually became shallower, the median grain size of the sand also rose slightly (fig. 6). It may be expected that

this process is coupled with a reduction in the concentration of the young spat; but as we said before, our data are not sufficiently complete to allow of comparing the spat-falls of the different years.

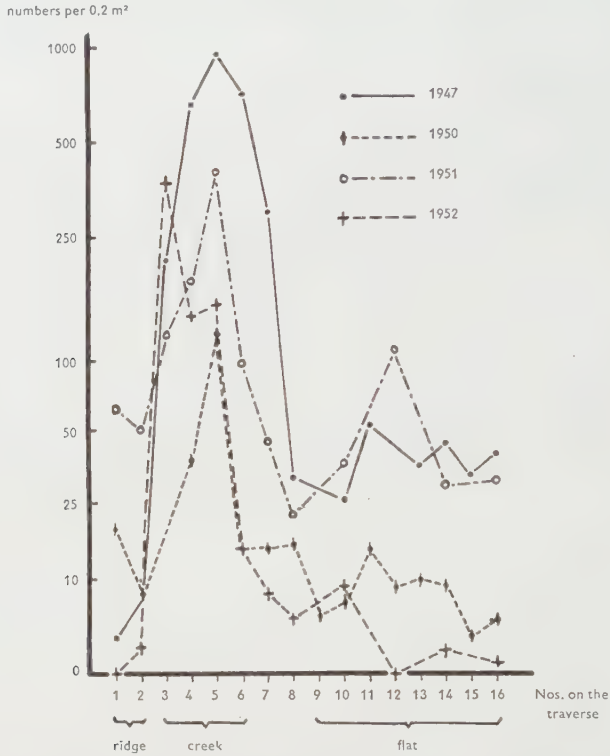

Fig. 5. Spat-fall on the Zeehondenplaat in 1947, 1950, 1951 and 1952 (in the year 1948, 1949, 1953, 1954 and 1955 the amount of spat was negligible).

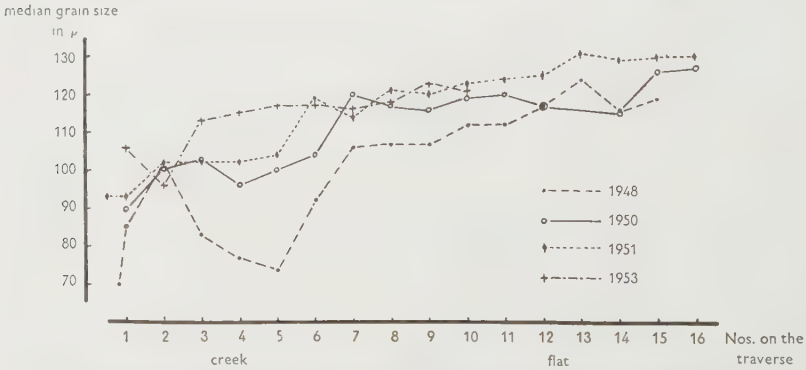

Fig. 6. Median grain size on the Zeehondenplaat from 1948 to 1953.

Also on other flats we invariably found a correlation between the density of the cockle-spat and the mud content of the substratum. In order to summarize the available data in a table, the samples from each mud-flat with relatively high numbers of cockle-spat were plotted against the samples with relatively little cockle-spat. After this, the average cockle density, the median grain size of the sand, and the average quantity of mud in the substratum were computed (table III).

TABLE III

Parts of tidal-flats with, respectively, low and high densities of cockle-spat, and the relevant structure of the bottom, expressed in the median grain size of the sand and the mud content.

date	name of tidal flat	sampling point	numbers of spat per 0.2 m^2	median grain size in μ	mud content in gr/l
9-9-1948	Zeehonden-plaat	7–16	30	113	4
		4–6	190	87	126
23-9-1948	Balgzand	1–6	1–2	148	46
		7–12	46.0	104	256
16-11-1948	Eastward part Zuidwal	2,3, 9–12	16.8	134	10
		4–8	320	129	47
22-9-1948	Westward part Zuidwal	2,3, 9–17	0.6	121	27
		4–8	16.2	105	58
8-9-1948	Paardenhoek	1–25	14.8	125	12
		26–32	152.4	115	28
5-9-1948	Staart van Schieringhals	1–5	20.2	87	9
		6–19	147.6	97	18
3-9-1948	Griender-waard	1–16, 25,26	0.9	136	9
		17–24, 27,28	33.3	137	11

Taking the above table as a whole, it will be seen at once that there exists no absolute correlation between the quantity of spat and the structure of the bottom. But if we look at the figures of each separate flat, we shall find, on the contrary, that there does exist a definite correlation. For, on 5 out of the 6 flats, the mud content of the bottom is considerably higher in those places where a large number of cockles are found, than where they are less numerous. We further see that, on 4 out of the 6 flats, the largest number of cockles are found in places where the sand is finest—but this correlation is probably hardly significant. The picture shown by the Grienderwaard deviates somewhat

from that of the other flats. This is probably the result of the dense bird population there, which affects the distribution of the cockles over the flat in a particular way (see p. 399).

Summarizing, we may say that the densest cockle population on a tidal flat will be found where its mud content is highest, and where, moreover, the sand has a slightly finer grain size. On the other hand, comparison between the flats as such does not provide any evidence of the existence of a correlation between the structure of the bottom and the density of the cockle population.

We furthermore collected, on the Zeehondenplaat, some data concerning the quantities of coarse organic matter. This detritus lies, at low tide, on the edge of the deep channels; but during flood it is dragged along over the flat, being re-transported back to the channel during ebb. This material was collected during flood, with the aid of glass jars dug down to the upper edge into the sand. The largest quantity of detritus could be deposited where the current was weakest. It was therefore to be expected that there would exist a close correlation between the quantity of detritus collected in this way, and the mud content of the substratum, a correlation which, in fact, does exist, as is shown in fig. 4.

It may be expected that there is a certain correlation between the quantity of detritus and the density of the cockle spat; and in fact, as fig. 4 shows, this correlation, too, exists, although not quite as distinctly. The reason for this is chiefly that the edge of the channel, which is so rich in detritus, does not appear to be a favourable habitat for cockles —as we already pointed out, and to which we shall revert again.

2. *Influence of temperature, salinity, light, and duration of submersion*

Looking now at other factors which may possibly be of influence on the occurrence of local differences in the density of the cockle population, we may leave both the temperature and the salinity out of consideration, because it appears to us that nowhere in the area investigated was any particular temperature or salinity ever found that might be expected to be injurious to the spat. An exception, in this respect, are those flats which fall dry for prolonged periods, and where conditions may therefore be extreme. We did find, in fact, that heavy rains may be fatal to the small spat (see p. 383); but this may, in our view, be regarded as an exceptional case.

It also seems improbable that the oxygen supply could have any effect. Even during prolonged dry periods the spat does not die, because it is capable of taking up oxygen also from the atmosphere (Thamdrup 1935, p. 63).

Neither does it seem any more probable that light could have any

influence on the occurrence of differences in density—at any rate as far as the differences on the flats are concerned. At most, we believe, the light might be of some influence on the cockles in deeper water, where its intensity, of course, is subject to considerable variation, owing to differences in depth and the degree of turbidity. We shall revert to this on p. 391.

Neither can the available quantity of food be regarded as responsible for the differences in the density of the spat; for these differences already arise before the spat has reached a length of 2 mm. In the course of subsequent months, the total weight of the spat, per unit of area, increases by a multiple; and evidently the available quantity of food is adequate for this increase. One can hardly assume, therefore, that the amount of food available was insufficient to ensure the survival of the very tiny spat. (Here we take the point of view that the composition of the food of the spat is not essentially different from that of the adult animals, as appears from a preliminary investigation.)

What we may assume, however, is that, on flats which fall dry for prolonged periods, the time during which the spat can take in food is too short. Indeed, it is hardly possible to imagine that there should not be, somewhere on the flat, a limit above which the spat cannot possibly maintain itself. And, in fact, we found, on two flats along the coast (Balgzand and Zuidwal), only few cockles in those places which are under water for less than about 4 hours per tide, while hardly a single cockle was found on the highest parts which are under water for only a couple of hours (see table IV).

TABLE IV

Number of cockles found on the Balgzand Flat, from high-tide to low-tide level, on September 17, 1947.

sample no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
submersion time in hrs.	11.5	±9	±8		±7	5		±4		3		2.5		1	0
number of cockles	17	44	50	59	56	23	17	12	15	3	3	2	2	0	0

Number of cockles found on the south-west part of the Zuidwal, on Sept. 20, 1947.

sample no.	1	2	3	4	5	6	7	8	9	10	11
submersion time in hrs.	9		8		6		4	3	1 ½	1	0
number of cockles	36	49	34	41	37	23	18	4	0	0	0

Neither did we find any cockles on the higher parts of the Ameland Wad, of the Frisian Wad near the Zwarte Haan, and of the Lauwers Sea Wad; but no submersion time of any of these flats was recorded.

We assume, therefore, that the near-absence of cockles on the highest parts of the flats must be attributed to the latter's prolonged emergence during the low water tide. However this may be, it cannot be due to any unduly high current velocity; for this is fairly low, precisely on the higher parts of these flats, so that, other things being equal, one would rather expect to find an accumulation of cockle spat in these places.

3. *Influence of biotic factors*

Next to abiotic, biotic factors too may have an influence. Thus, it appears that the spat's distribution is affected, before its final settlement, by the presence, on the tidal flats, of other animals, especially of adult cockles. This could not have been the case in the exceptional year 1947 (see Introduction); but during the following years we were frequently struck by the fact that, in areas harbouring a large number of adult cockles, a relatively small proportion of very tiny spat could be found. The clearest indication of this phenomenon was found on the Zuidwal in June 1949, where spat of 1 mm was practically not present at all in those areas which contained large numbers of adult cockles (see table v).

TABLE V

Distribution of old and young cockles on the Zuidwal tidal flat on June 29, 1949.

point	1	2	3	4	5	6	7	8	9	10	11	12	13
numbers of adult cockles per 0.2 m^2	0	0	51	430	570	266	81	44	23	2	4	5	0
numbers of cockles of 1 mm	27	48	102	8	2	6	33	46	38	29	17	25	0
numbers of cockles of 2–3 mm	5	9	35	86	41	52	12	10	5	16	21	7	0

Kreger (1940, p. 177) observed a similar phenomenon, on the same flat. But he rejected the suggestion that the adult cockles obstructed the settlement of the spat, on the ground that, within a number of sectors of 1/4 m^2, from which all adult cockles had been removed, no more spat settled than outside these sectors, where a dense cockle population was living. Since we now know, however, that spat smaller than 2 mm length is readily transported in quantity, it is understandable that no

larger amounts of spat were found in these small sectors without large cockles, than in the surroundings. KREGER's experiment, therefore, does not prove that the presence of old cockles has no effect whatever on the settlement of the spat.

We accordingly conceived the possibility that the very small spat, which is driven up by the currents to places with a very dense population of adult cockles (i.e. some hundreds of specimens per 0.2 m^2) runs a considerable risk of being "inhaled" by an old cockle. In order to demonstrate the plausibility of this hypothesis we made a number of laboratory experiments in June 1949. In a shallow tank with running sea water a number of adult cockles of between 2 and 3 cm length were given an opportunity to dig themselves into a layer of sand. After this, in daylight, we let one small cockle (0.5–3 mm length) at a time out of a pipette at about 1 cm upstream of the siphon of one of the large cockles. The current carried the small cockle close to the big cockle's siphon. At the first touch of the siphon-tentacles, the large cockle usually closed its shell, thereby preventing the small cockle from being sucked in. But in some cases the big cockle appeared to have closed itself too late, so that the small animal had already been sucked in. In that case, the large cockle soon opened its siphon again, and forcibly squirted the intruder out through the inhalant-siphon. These exhaled small cockles —14 in all—were collected with the aid of a pipette, and examined in a separate dish. We found that those that had been inhaled by the adult cockles were exclusively very small specimens. They measured between 600 and 900 μ, while the average size of the 40 young cockles used for this experiment was about 2 mm. Evidently, therefore, only the very smallest cockles, less than 1 mm long, can be inhaled. The experiments yielded no data whatever concerning the *chance* of such small cockles to be sucked in, when finding themselves in the vicinity of a large cockle's siphon. Neither were the majority of the very smallest animals inhaled; it is, however, probable that the smaller the spat, the larger will be the percentage inhaled.

It was further found that, after inhalation and discharge, all the small cockles were more or less covered with flakes of slime. They made violent efforts with their feet to crawl into the sand, but the slimy coat proved a considerable obstruction. Next day, 12 out of the 14 specimens were found dead, either on or in the sand, only two remaining alive. Among the cockles that had not been inhaled, on the other hand, mortality was practically nil.

There is no doubt, therefore, that on the tidal flat inhalation of spat by older cockles also occurs. The small spat squirted out again will then be washed away by the current, and probably die elsewhere. Our experiments yielded no data to justify any conclusion as to the extent

to which the process of inhalation can quantitatively affect the settlement of the young spat; but we are surely justified in assuming that this process may be of some significance.

It will be seen from table v that the slightly larger cockles—between 2 and 3 mm—, in contrast to the tiny, 1 mm-long animals, occur most densely in precisely those places where the population of old cockles, too, is extremely dense. The explanation of this fact might be as follows. The location where large numbers of old cockles are present is, as such, favourable for the settlement of the spat, but so long as the spat is inhaled by old cockles, settlement remains impossible. Only spat large enough to escape inhalation (>1 mm), and small enough to be transported by the current (<2 mm), therefore, will successfully settle in those places. Among a very dense cockle population, therefore, the spat-fall will consist mainly of specimens between 1 and 2 mm.

The influence of the older year-classes on the distribution pattern of the spat is, in general, only small. This would appear to be due, first of all, to the fact that the older populations are rarely dense enough to prevent the settlement of the small spat, and secondly to the fact that the population, however dense it may be, cannot have much influence on it from the moment the spat has reached a length of 1 mm.

Summarizing, our conclusion is that, on low flats, emerging for less than 6 hours per tide, the differences in density of the cockle populations can be explained largely by the degree of shelter which the flat provides locally against the transporting action of the current. But the absence of cockles in the channels is determined by other factors. Further, the population density of cockles on flats which generally emerge for longer than 4 hours per tide does not primarily depend on the velocity of the current, but is probably affected chiefly by lack of food, due to the short submersion time. Finally, a very dense old population may have an adverse effect on the settlement of the very youngest spat.

B. CHANGE IN DENSITY AFTER FINAL SETTLEMENT

It is a wellknown fact that there is a very considerable decrease, particularly during the first year, in the density of a cockle population. This was observed in the Wadden area by, amongst others, Thamdrup (1935, p. 88), Wohlenberg (1937, p. 41), Kreger (1940, p. 190), and Smidt (1951, p. 81–84). The method to determine the decrease in density invariably consisted in determining, at certain intervals varying between a few weeks and several months, and in more or less the same places, the density of the local cockle population. Since none of the above investigators continued their observations for more than 1–2

years, it is impossible to calculate from their data by what percentage a given cockle population annually diminishes in density.

Our own data, relating to the Zeehondenplaat, show a rapid diminution in density for the year-class 1947 (cf. fig. 7): from the first late

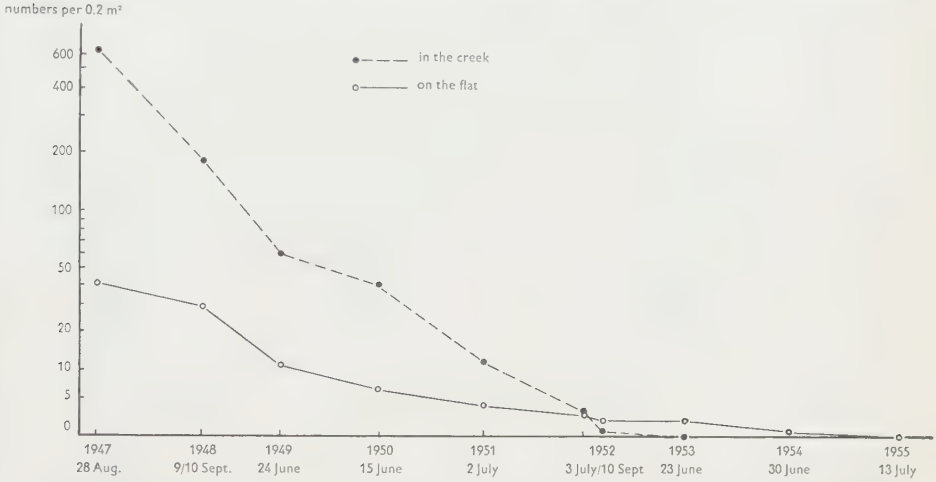

Fig. 7. Number of cockles per 0.2 m² of the year-class 1947 on the Zeehondenplaat.

summer until the third summer the numbers found decreased very considerably year after year; after this period the annual decrease was less. In addition, there appeared to be an important difference between the cockles on the flat and those in the shallow creek: the reduction in the much more densely populated creek exceeds that on the flat, so that, after 5 years, the flat is more densely populated than the creek.

Expressing the annual diminution as a percentage of the number of cockles present in the preceding year (see table VI), we find that the yearly decrease on the Zeehondenplaat varies between 26 % and 61 % (yearly average 36 %) on the flat; in the shallow creek, on the other hand, between 34 % and 80 % (average 65 %).

TABLE VI

Annual decrease in density of the cockle population on the Zeehondenplaat, expressed as percentages of the density in the preceding year

	on the flat, nos. 10–16	in the creek, nos. 3–6
Aug. 1947–Sept. 1948	26	71
Sept. 1948–June 1949	61	66
June 1949–July 1950	42	34
July 1950–July 1951	31	80
July 1951–June 1952	25	64
June 1952–June 1953	33	75

As regards the Zeehondenplaat, therefore, we find that the annual diminution in density is relatively great where the place of settlement is favourable; while, in places where little spat has settled, the annual reduction is less. This unequal decrease causes a change in the distribution of the population over the flat as a whole; that is to say, it levels out the differences. We shall return further on to the question why the reduction in the creek exceeds that on the flat.

We do not have a similarly exact picture of the diminution in density on any other tidal flat; but the general impression we received on the ground of our data concerning other flats confirms the picture presented by the Zeehondenplaat; viz. the denser the population, the greater the yearly decrease in density. In table VII we give, for several flats (including the Zeehondenplaat), the figures of the average annual decrease of the population density in the years 1947–1949.

TABLE VII

Density and mortality of the cockle population in different parts of the Wadden Sea

	average starting density per 0.2 m^2	percentage of annual mortality
Creek of Zeehondenplaat	402	69
South part of the Staart van Schieringhals	131	42
Zuidwal	113	48
Paardenhoek	53	27
Flat of Zeehondenplaat	36	44
North part of the Staart van Schieringhals	34	10

The tidal flat south of Griend presented quite a unique picture. Immediately to the south of the island there is a small, shallow creek, in which fairly large numbers of cockle spat (up to ca. 500 per m^2) were found in August 1947; but one year later these cockles had completely disappeared. The fairly numerous spat which fell there in 1948, too, was found to have totally disappeared twelve months later. During those years, however, no such fast disappearance of cockles was observed anywhere else.

Changes in the density of a cockle population may be due to two causes. First, the cockles may be washed away, and displaced to other parts of the flat, thus altering the density locally. Secondly, of course, mortality is bound to affect the density.

a. Displacements

As already stated, the observations of Miss BAGGERMAN (1953) provide clear evidence of the fact that the young cockle spat up to a size of nearly 2 mm is still transported in quantity by the tidal currents,

whereas this is no longer the case with spat larger than 2 mm. Her data concerning this slightly larger spat are not numerous; but we have other data of our own which confirm that larger spat is hardly, if at all, transported. This may be seen, for instance, in fig. 8, where in the upper columns, the size-distribution of the cockle spat on the top of the

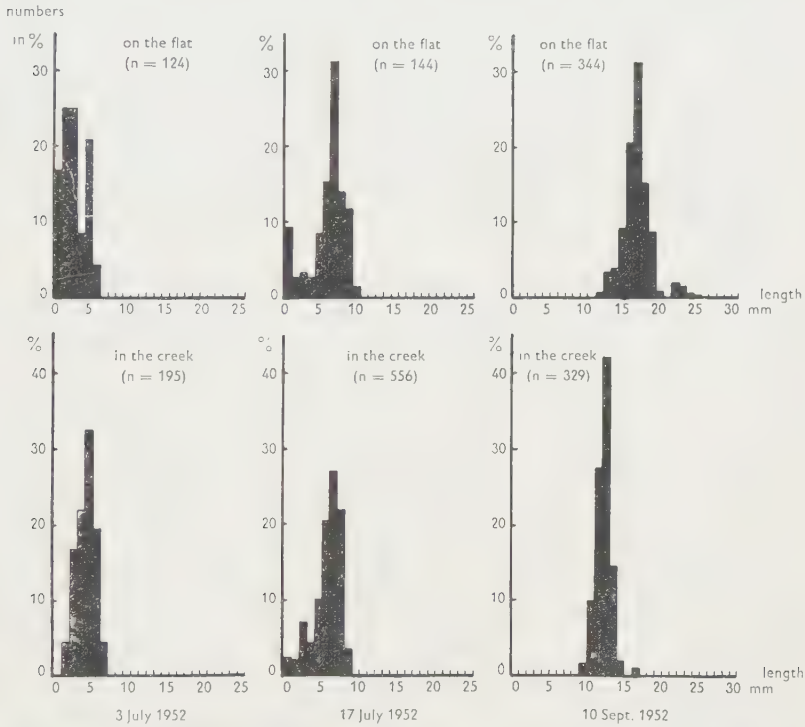

Fig. 8. Diagrams of length of the spat in 1952, on July 3, July 17 and Sept. 10, on the flat and in the creek of the Zeehondenplaat. (In parentheses the number of cockles measured.)

Zeehondenplaat is indicated, and in the bottom columns the size-distribution in the creek. In the autumn (10 Sept.) we find the same size-distribution as that invariably shown by the older cockles; i.e. small animals in the creek; large animals on the flat. This is because the conditions for growth are more favourable on the flat than in the creek, as we shall see further on. But earlier in the summer, viz. on July 3 and on July 17, this difference in the size of the cockles was not yet found to exist; nay, in the beginning those in the creek were even, on the average, distinctly larger than those on the flat. We observed the same phenomenon also in other years; not until about August does the spat become larger on the flat than in the creek.

We can find only one explanation of these facts—taking as our starting point that the small spat of under 2 mm is transported across the flat over and over again. There is no doubt that the chances of being washed away once more by the next flood, and being lost in the process, are smallest in the sheltered condition of the creek. It is there that the spat accordingly has the best chance to grow; and this explains why, in the beginning, the creek is relatively rich in the category of the largest spat (5 mm). But two months later the length of the cockles on the flat is found to exceed that of the cockles in the creek quite distinctly. This would not have been possible if, during this intermediate period, the cockles had still been exposed to being washed away and transported.

It appears likely, therefore, that no interchange of any significance occurred, after July of the first summer, between the cockle spat on the flat and that in the creek.

On the Zuidwal, too, we found, in two successive years, that the distribution of the smallest spat over the flat deviated somewhat from that of the 2 mm spat. We accordingly assume that the distribution of the 2 mm spat determines the distribution of the older spat.

We have found, therefore, that cockles longer than 2 mm are apparently scarcely displaced. None the less, it seems worth while to ascertain whether any displacement of the animals, after this time, must be regarded as completely precluded. Data communicated by Stephen (1931, p. 290, and 1932, p. 62–63), Thamdrup (1935, p. 74–75), Kreger (1940, p. 166–168, 174–175) and Smidt (1951, p. 81–84) suggest that this is, in fact, the case. But neither would one have suspected, on the ground of these authors' data, that the animals smaller than 2 mm would be subject to displacement. Evidently, the cockle population of the Zeehondenplaat is particularly suitable for getting an insight into the question of transportation, because of the great local differences in density and rate of growth occurring there. We shall, therefore, endeavour to ascertain if, and if so to what extent, there has been an interchange of cockles between the flat and the creek. The average length of the cockles already differs considerably on the flat and in the creek in the first year (1947). Consequently these cockles will have a difference in size of the first winter-ring at any later age. Since the winter-rings remain distinctly recognizable through the years, it is possible to determine, for each separate sample area, and from year to year, the average size of the first winter-ring. If, within a given population, there has been no displacement of cockles in the course of the years, then the average length of the winter-ring must retain the same value for each separate sampling area.

In fact, as fig. 9 shows, the length of the winter-rings in 1948 presents more or less the same picture as the length of the cockles in August

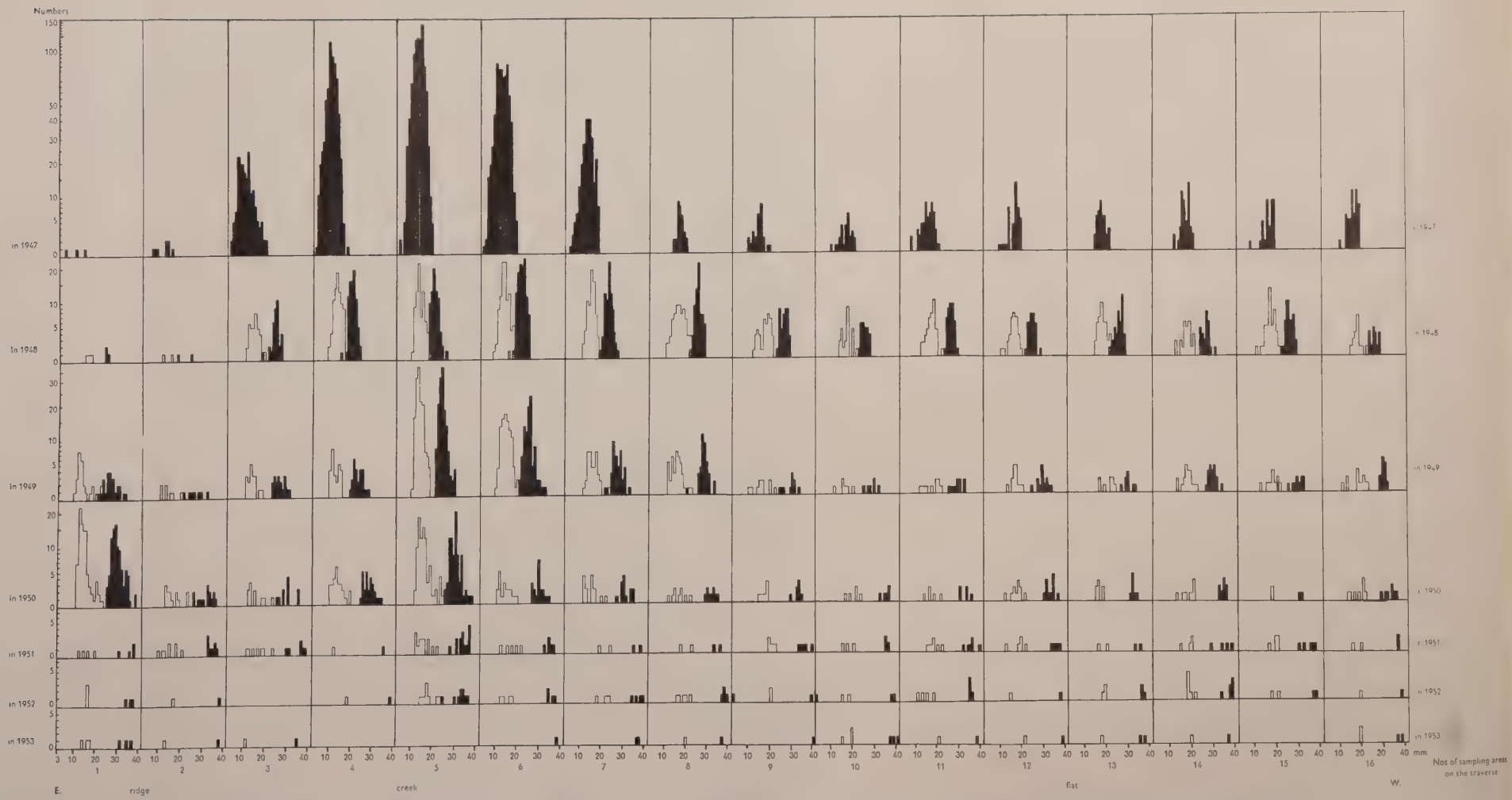

Fig. 10. Distribution of the cockle year-class 1947 over the Zeehondenplaat from 1947 to 1953. The numbers of the diagrams (1–16) are the numbers of the sampling areas on the traverse from east to west. The abscissa of each diagram has a millimetre scale running from 3 to 41 mm. Black squares show the frequency distribution of length of the cockles of each sampling area (0.2m²) in each year; blank squares, the frequency distribution of the first winter-ring.

1947. Of course, the linear measures for August 1947 are slightly lower, because the summer growth of the cockles had not yet ended by then.

The fairly great local differences in length of the first winter-ring as between the points of the traverse (see fig. 10) oblige one to exercise the necessary caution in comparing the length in 1947 with the winter-rings in subsequent years. This is the more so because the sample-points were

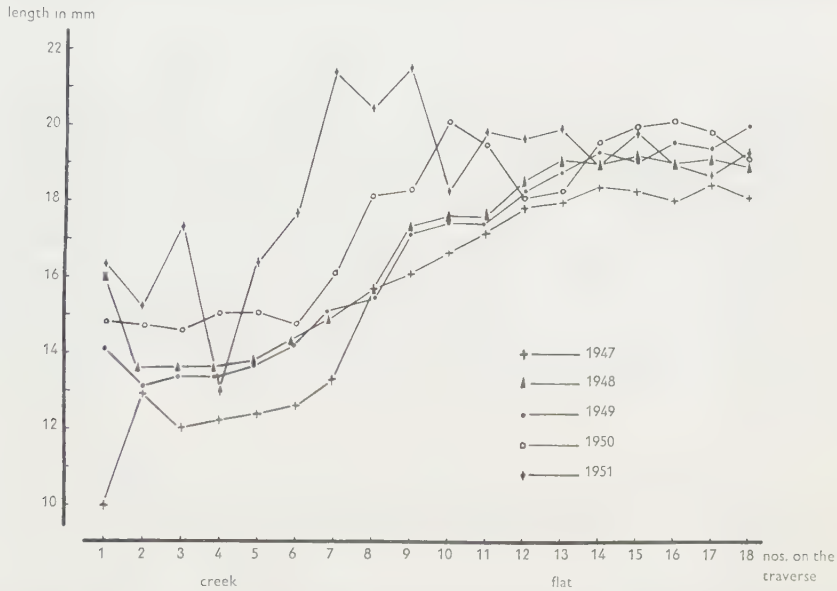

Fig. 9. Length of the cockle year-class 1947 on the Zeehondenplaat in September 1947, and the length of the first winter-ring of these cockles in the years 1948–1951.

not completely identical during the successive years, while the numbers of cockles measured, especially in later years (e.g. 1951) were very small, as fig. 10 shows. Taking this into account, the conclusion is justified that the distribution of the average length of the winter-rings over the traverse does not deviate essentially, for 1948 and 1949, from the distribution of the average length over the traverse in August 1947. To meet the objection of too small numbers we can determine, for each year, the average length of the first winter-ring of the cockles on the top of the flat (Nos. 10–18) and that of the cockles in the creek (Nos. 3–6, see table VIII). It will then be even more evident that there is no difference, and that the cockles are not displaced very much during the first three years.

Not until 1950 and succeeding years does the length-distribution of the winter-ring averages appear to become much more capricious. It is then, therefore, that displacement must have occurred. This is also

TABLE VIII

Length of the cockle year-class 1947 in August 1947, and length of the first winter-ring of this year-class, as measured in successive years.

	on the flat	number measured	in the creek	number measured
length in Aug. 1947	17.3 mm	281 spec.	12.3 mm	2299 spec.
first winter-ring, measured in :				
1948	19.0 mm	205 spec.	13.6 mm	195 spec.
1949	19.1 mm	82 spec.	13.6 mm	167 spec.
1950	19.0 mm	50 spec.	14.9 mm	133 spec.
1951	19.2 mm	36 spec.	15.9 mm	27 spec.

evident if we compare the frequency distribution of the cockle length as between the different years (see fig. 10). At the edge of the flat, along the big channel (near sample No. 1), there were, from 1949 onwards, many more cockles than previously; and these—to judge by their small winter-rings—had originated from the small creek (near 3–6), at any rate for the most part. In the creek itself, on the other hand, there were, from 1950 onwards, cockles with such large winter-rings that they had evidently originated from the flat. On the top of the flat, on the contrary, no considerable changes in the length of the first winter-rings are to be found.

Here, therefore, is the total picture we can form of the displacement of the cockles on the Zeehondenplaat: the youngest spat —up to 2 mm —is transported in quantity by the tidal current; the larger spat, on the contrary, is no longer transported. Neither are there any considerable displacements during the first winter; nor during the second winter, when only a few cockles have been displaced from the creek (Nos. 3–6) eastward towards the channel. But displacement occurs ever more distinctly from the third winter onwards; chiefly in an eastward direction. While the population in the creek had a winter-ring of 13.6 mm in 1948 and 1949, the average length of this ring was 14.9 mm in 1950. It may be calculated from this that, in 1950, the number of cockles in the creek, that had come from higher up the flat, must have been *at least* 22 %—a percentage which, as is evident from fig. 10, further rose steadily during succeeding years.

Now how can we explain the fact that it is precisely the recently fallen spat *and* the three-year-and-older animals that are transported, but not the one- and two-year-old cockles? One might suppose that weather conditions in the years after 1949 were unfavourable; but this is improbable as regards 1951, 1952 and 1953, because at that time the younger year-class 1951 was of an exceptionnally stay-at-home type, as will be seen from the first winter-ring of this year-class (see table IX). Moreover, the few data which we have about the third year of the

TABLE IX

Length of the first winter-ring of the cockle year-class 1951 on the Zeehondenplaat in successive years

	on the flat	number	in the creek	number
1952	17.8 mm	187 spec.	13.0 mm	837 spec.
1953	18.0 mm	146 spec.	13.1 mm	1056 spec.
1954	17.6 mm	7 spec.	15.9 mm	12 spec.

year-class 1951 point to the fact that these animals, too, were no longer so stationary in their third year: out of 12 cockles collected from the creek in 1954, three specimens had a winter-ring of more than 18 mm, so that they cannot possibly have been autochthonous creek-cockles.

We assume, therefore, that it is not due to incidental environmental conditions that the one- and two-year-old cockles change place so little. What, then, may be the real cause? We believe it must be sought in the greater speed with which cockles of that age are able to dig themselves into the sand.

In order to demonstrate this, cockles of different size-categories were dug out and laid flat on the sand bottom, covered by about 4 cm water.

TABLE X

Digging-in times of cockles placed on the sand, covered by water

size (min.-max.)	number	digging-in time (min.-max.)
water temperature ± 16°C		
2.4 mm (2–3 mm)	28 specimens	8 sec. (2–16 sec.)
14.9 mm (10–19 mm)	11 specimens	2.2 sec. (15 sec.–7 min.)
31.4 mm (29–35 mm)	12 specimens	6 min. (2–15 min.)
water temperature 10.2°C		
13.4 mm (11–16 mm)	32 specimens	8.6 min. (3–15 min.)
27.2 mm (25–29 mm)	5 specimens	14.2 min. (7–26 min.)
water temperature 6.4°C		
12.5 mm (10–16 mm)	11 specimens	17 min. (7–30 min.)
27.8 mm (26–30 mm)	5 specimens	25.4 min. (11–41 min.)
water temperature 2.5°C		
13 mm (10–16 mm)	37 specimens	(digging in not yet started
26.0 mm (25–28 mm)	5 specimens	after 30 minutes)

After this the time was noted that passed between the first protrusion of the foot and the moment when the cockle completely disappeared under the sand. These observations were made on the Zeehondenplaat in July 1950, at a water temperature of, roughly, 16°C, and on the Zuidwal in March 1955. On the Zuidwal the temperature of the water in the pools on the flat varied considerably, according to the depth of the pool and the distance to the low-water level, so that observations could be made at 10.2°, 6.4°, and 2.5°C.

As the figures in table x clearly show, the digging-in time is dependent upon the size of the cockles and the temperature of the water. In summer it is the *spat* which digs itself in most rapidly; the three-year-older animals most slowly. In winter, the six-month-old animals dig themselves in faster than the eighteen-month-old ones (older cockles were unfortunately not available). At very low temperatures—2.5°C—not a single cockle made any show of going to dig itself in. To make sure that these cockles might not, perhaps, be in bad condition, they were transferred for control to water at 10°C. At this higher temperature they disappeared at about the same rate as the cockles that had been tested at 10.2°C.

These data support our assumption that the speed of digging in is an important factor with respect to the extent to which the cockles are washed away and displaced. At temperatures of ca. 6°C the danger of being washed away will be especially considerable for the largest cockles; at still lower temperatures this danger is equally great for the smaller cockles as well. The few times when we witnessed cockles of all sizes being washed away it was, in fact, precisely at very low temperatures (see pp. 379 and 388).

Since we may assume, therefore, that a cockle, once it is in an exposed position, can escape being transported by quickly digging itself in, the question is whether, in that case, the very small spat—which is transported in such quantities—is *not* capable of digging in rapidly. As we observed, however, this small spat of less than 2 mm digs itself in extremely fast; definitely not less fast than the 2–3 mm spat. But the very smallest spat gets to such slight depth—2–3 sand-grains deep—that it can be readily washed away despite its great digging-in speed.

It is not only on the Zeehondenplaat that the one- and two-year-old cockles are found to be fairly stationary; this is also the case on other tidal flats. Although we have no quantitatively satisfactory data concerning the yearly displacements of the cockles on other flats, the variations in size of the first winter-ring in the course of a few years nevertheless provide us with a picture of the extent of displacement of the cockles, providing the local differences in the rate of growth are great enough. These differences, in fact, were maintained from one

winter to the next; at any rate on two other flats—as will be seen from table XI.

TABLE XI

Length in mm of the first winter-ring in successive years. Space between samples: 50 metres

	sample	1	2	3	4
East part of Zuidwal	1947	17.0	14.8	13.4	14.8
	1948	16.7	14.0	13.7	14.6
	1949	17.0	14.1	13.9	14.7
West part of the Staart van Schieringhals	1948	16.7	17.2	18.1	18.5
	1949	16.9	17.2	18.3	18.4

Only one case came to our notice, during the whole of our investigation, in which the entire cockle population became mixed up over practically the whole area, from the low-water level to the high-water level, in the first winter of its life. Since, moreover, the total number of cockles had increased in the course of this winter, there must have been, in this area, an immigration of cockles from other parts of the flat (table XII). This was on the Balgzand, where the bottom was subjected to considerable changes caused by dredging work being done in the vicinity, which greatly increased the amount of mud on the flat. For that matter, without further factual data it is not clear what may be the causal connexion between an increase of mud on a flat and the displacement of cockles.

TABLE XII

Length and density of the cockles on Balgzand in 1947, compared to the length of the 1st winter-ring and the density in 1948. Space between samples: 50 metres

sample no.	1	2	3	4
length of cockles in mm in Sept. 1947	13.5	12.1	11.5	10.2
length of 1st winter-ring in Sept. 1948	17.0	15.0	14.9	15.0
numbers of specimens per 0.2 m^2 in Sept. 1947	2	119	46	23
numbers of specimens per 0.2 m^2 in Sept. 1948	13	164	85	22

On February 28, 1948 we observed how, in winter-time, during an ice-drift, cockles can be washed away and transported. This was on the

Zuidwal, where there was an ice-barrier along the low-water level. This caused a number of shallow runnels to be ground into the flat, which were perceptibly widened and/or shifted elsewhere. Over large parts of the flat a layer of sand had been washed away, as could be observed from the presence of *Lanice*-tubes protruding as far as 4 cm above the surface of the bottom. There were cockles all over the place—by then about 3–4 years old—, washed up and exposed in these creeks, in some places thousands per m^2; here and there they were dragged along, as the cold prevented them from digging in quickly. We dug up twenty cockles out of the sand, and put them down, but during the 7–10 minutes that we watched them we noticed not the slightest movement —just as we had expected. None the less, they must have been able to dig themselves in during those days, for we found many cockles dug in everywhere among the 4 cm-deep washed-out *Lanice*-tubes. Where the washing-out process had started quite recently, however, the cockles were still lying exposed. Probably they could not dig themselves in again until the slightly warmer flood water began to flow over the flat.

Another question is, why the cockles on a flat such as the Zeehondenplaat should be displaced exclusively in an eastward direction. If our conception is correct, i.e. if the transport of the cockle is a passive process, we may assume that the greater part of the transport will take place when the wash of the waves moves the upper layer of the bottom, while the current, moreover, is so powerful that a relatively heavy object as a cockle is dragged along.

Our observations on the Zeehondenplaat give us reason to suppose that, under normal weather conditions, the tidal current is not capable of washing away adult cockles. But it may be assumed that, when strong westerly winds sweep the tidal current across the flat, while the waves stir up the bottom, even large cockles may be washed up and exposed. One might even expect a still greater effect to be caused by an ebb-tide *against* a westerly wind; but it is understandable—as well as evident from observations on the locality—that the water, under those circumstances, is blown in a more or less eastward direction during the whole of the tidal period.

Now that we know the extent to which the cockles are displaced in the course of time, we may revert to the question which we put at the beginning of the present chapter; viz. to what extent the annual decrease in population density can be a result of the displacement of cockles. The answer to this question is clear, as far as the Zeehondenplaat is concerned. There, it is found that the number of animals displaced eastward from the flat to the creek forms a mere fraction of the annual decrease in population on this flat. A relatively large number of animals was transported eastward from the creek to the edge of the

channel; as against this, the population in the creek was supplemented from the higher part of the flat; and from 1950 to 1952 the flat-cockles increasingly predominated in numbers. The decrease in numbers of the autochthonous creek-cockles, therefore, is still greater than fig. 7 might lead one to suppose.(see p. 21)

We have no direct observations, with respect to transportation, concerning other tidal flats in the Wadden Sea. But one may expect—in view of our results on the Zeehondenplaat—that the more a flat is exposed to the wind and the current, the greater will be the influence of the displacement of cockles on the annual changes in population density. In the area investigated we can point to only a couple of flats that are more exposed than the Zeehondenplaat; viz. the Jacobsruggen, and the Staart van Schieringhals. There, the displacement of cockles will probably have a greater effect on the density than is the case on the Zeehondenplaat; but on other flats this effect will, on the contrary, be smaller rather than greater.

b. Mortality

As we remarked above, part of the cockles disappeared from the area investigated. Of some of these cockles, the shell may be left behind in the bottom on the spot. And in fact, one here and there finds—especially in sheltered places—empty shells; but that is an exception. The great majority of these dead cockles, it appears, are washed away (either before, during, or after death);finishing up, according to the profile of the flat and its position with respect to wind and current, either higher up on the flat at high water level, or, on the contrary, lower down, for instance in a creek. Thus we found, close to the Zuidwal, quite a bank of shells along a stone dam on the south-east side of this flat, and, in the Lauwerszee, dense masses of shells at high water level against the salting.

The cockle shells from flats situated lower down, however, will generally finish up in a channel. If a powerful current runs through this channel, these shells will be dragged along elsewhere; but if the current in the channel is a weak one, the shells will remain lying by the side of the bank. Thus, we found, in the Zuidoostrak, on a muddy bottom, large numbers of shells which, we assume, originated from the adjacent flat, the Paardenhoek. In the muddy parts of the Oude Vlie we found cockle shells that had surely come from the Zeehondenplaat, and also in the muddy Vlieter we found cockle shells which had definitely originated from the near-by Lutjeswaard.

Since we know from the preceding chapter that on the Zeehondenplaat the annual decrease in population density is affected only to a

slight degree by displacements, we are justified in regarding the curves in fig. 7 mainly as mortality curves. During the first years (1947–1949) the mortality in the creek is realtively higher than on the top of the flat (see table VI, p.371). This also appears to be the case in the later years (1950–1952); for, as fig. 10 clearly shows, the number of animals on the Zeehondenplaat with a small winter-ring (i.e. originated from the creek) constantly diminishes more than the number of animals with a large winter-ring (i.e. those from the flat). In September 1952, indeed, no more cockles originating from that creek could be found anywhere; those few that were found in the creek hailed from the flat. This is not to say that, in the creek, the small creek-cockles have a shorter life than the large flat-cockles transported thither; for it is quite possible that *all* cockles soon die in the creek. We have other indications to the effect that, wherever the conditions for growth are favourable, the cockles live long, while under unfavourable conditions for growth they die young. As we shall see further on, cockles with the most rapid growth are found in the Atlantic coastal water west of Scotland; and there, according to WALTON (1920), they reach the age of 7 years and even older. On the other hand, the majority of cockles in places where they grow definitely slowly, do not, according to our observations, get older than 1–3 years. On those grounds where the cockles grow rapidly, e.g. on the Paardenhoek and in the North Sea off Callantsoog, we found as late as 1953 a relatively large number of cockles of the spat-year 1947. As against this, we found in the same year, in places where growth is a slow process—as in the Lauwerszee and on the Balgzand—not a single cockle older than 2 years.

We revert further on to the causes of the fact that mortality increases as the cockles grow more slowly.

We shall now deal successively with the various factors which may possibly have an influence on the mortality of the cockle. Some of the factors to be discussed will primarily affect the growth of the cockle, and the mortality only secondarily. In such cases we shall refer to the part dealing with the animals' growth.

1. *Influence of the duration of submersion*

There is no doubt that there will be a limit somewhere, beyond which cockles cannot stick it any longer on the flat, because its period of emergence is too long. One may readily assume, however, that the young spat is more sensitive in this respect than older cockles. We do not believe, therefore, that later on the time of emergence is of much importance to mortality. None the the less, mortality is higher on flats situated high up than on low-lying parts. This, however, we attribute

to the fact that, during emergence, other factors may sometimes reach extreme values. This indirect influence of submersion wil be discussed below, under the different factors involved.

2. *Influence of salinity*

It is quite certain that the salinity of the water can be of influence on the mortality. Nothing is known so far about the maximum salinity which the cockle is able to tolerate. In the Caspian Sea *Cardium edule* is still found at a salinity of 50.46°/₀₀ (BIRSTEIN 1936); but it is not certain that this *Cardium*-population has the same physiological demands of its environment as the West-European *Cardium edule*.

More is known concerning the minimum salinity in which cockles can go on living. In the former Zuyderzee they were found as far down as the area along the Zuidwal, where the salinity was as low as 10 °/₀₀ and even less (HAVINGA 1922). None of the cockles from this area—which have been preserved in the State Museum for Natural History, Leyden, and the Zoological Museum, Amsterdam—was found to be older than about 3 years, to judge from the number of winter-rings which we counted on about a hundred of these shells. Evidently their lives were only short.

As compared to the former Zuyderzee, the local differences in salinity found in the Wadden Sea are so slight that it seems improbable that they can be of any influence on an euryhaline species such as the cockle, in the locations investigated there.

It might perhaps be thought possible that, on the flats, the animals' life is threatened by temporary differences, on the one side, by too low salinities, e.g. after heavy downpours, and on the other side, by too high salinities as a result of evaporation. And in fact, as we found on July 2, 1953, on the Balgzand, heavy rains can be fatal to the *young* spat at any rate. Torrential rains had poured a layer of fresh water on to the high, horizontal part of the Balgzand; and before the flood reached this part of the flat, we observed the smallest cockles (1–2 mm) lying on the sand with their shells wide open. The larger spat and the older cockles were evidently less troubled, and normally dug themselves in when they were placed on the surface of the sand.

Nothing is known concerning the resistance of cockles to high salinity resulting from evaporation of the water during low-water periods. On a flat such as the Zeehondenplaat—where mortality is lowest precisely on the highest parts of the flat—the cockles were evidently unaffected by any possible differences in salinity.

3. *Influence of temperature*

As is wellknown, the temperature can affect the density of a cockle population in a spectacular manner. In winter, during periods of severe cold, entire cockle populations may die out. Observations to this effect are known from Britain, Scandinavia and Germany; SMIDT (1944) gives a survey of this effect of ice-bound winters. In the area we investigated, too, winter cold can exercise considerable influence. During a severe winter, such as the 1946–'47 one, practically all cockles died on the emerging flats in the western Wadden Sea. Although Dr. G. W. HARMSEN (oral communication) still found live cockles on the Grienderwaard in April, 1947—shortly after the severe frost—, we ourselves did not find more than one or two cockles anywhere on the tidal flats after that winter. For that matter, even on the Grienderwaard the surviving cockles evidently disappeared; for in September 1947 we failed to find, in the location indicated by Dr. HARMSEN, any cockles over one year old.

But also during less severe winters mortality may be considerable. Thus, during the brief period of frost in February 1954, we were surprised to find that the cockles had died over a very wide area, with the exception of only a few places where they had survived.

Since nothing much is known concerning the causes of death of the

TABLE XIII

Results of experiments in which cockles were temporarily exposed to low temperatures

numbers of cockles	temperature in °C during experiment	max. temp. in C.	min. temp. in C°.	duration of experiment in hours	time for raising the temperature	numbers of specimens that did not dig in	numbers of specimens that died	fatal period in days	max. duration of decease in days	min. duration of decease in days
35	0	+1.0	—0.3	24	1–2	0	0	—	—	—
12	—0.8	+0.1	—1.7	24	1	0	0	0	—	—
12	—1.2	0.0	—1.3	24	1	0	0	—	—	—
15	—1.6	—0.1	—1.9	24	1	0	0	—	—	—
30	—1.7	—0.4	—1.9	24	1–2	0	1	26	26	26
16	—1.9	—1.6	—1.9	6	2	2	3	35	51	15
16	—1.9	—1.7	—1.9	12	2	12	15	41	57	14
15	—1.9	—1.7	—2.0	24	2	all	13	26	51	10
10	—2.4	—2.0	—2.7	6	2	all	8	38	60	17
12	—2.5	—2.1	—2.8	12	2	all	10	13	20	9
12	—2.4	—2.1	—2.8	24	2	all	all	8	12	4
12	—6.7	—5.9	—7.2	6	2	all	all	17	36	3
12	—6.8	—6.1	—8.4	12	5	all	all	10	17	4
12	—15.9	—14.3	—17.1	6	6	all	all	0.5	5	0
10	—15.9	—14.3	—17.1	12	5	all	all	0.5	3	0

cockle during frosty periods, we made a number of experiments on this subject in the first few months of 1948 and 1949.

A large number of cockles of 20–30 mm were collected during the previous winter, and kept in an aquarium at a temperature of 3–4°C. Only those animals that completely dug themselves into the sand were used for the experiment. In each experiment 10 or more animals were transferred from the aquarium to an ice-box and placed, dry, on a dish. After this the temperature in the ice-box was gradually lowered in the course of one or a few hours, down to a certain value, which was then maintained as well as possible. The maximal deviations were recorded with the aid of a thermometer registering maximum and minimum temperatures. After a certain time, which varied between 6 and 24 hours, we raised the temperature in the ice-chest, in the course of a few hours, to + 3°C; after this the cockles were returned to an aquarium with a water temperature of between 3 and 5°C. During the subsequent two months we regularly recorded the proportion of cockles that had finally dug themselves in again, as well as each separate case of death (see table XIII, p. 384).

From the data obtained in this way it appears first of all that temperatures from 0° to —1.7°C are tolerated for 24 hours in succession without doing any harm. Admittedly it was a long time, in some cases, before the animals dug themselves in again after the experiment; but the individual differences in digging-in speed at 3–4°C are normally so great that we ought to have had a far greater quantity of material at our disposal to be justified in asserting definitely that the animals dig themselves in more slowly after a spell in the cold.

When cockles were exposed to temperatures of —1.9°C or lower, it was found that a proportion of them refrained from digging in afterwards altogether. They did move a bit at first, but their muscular contractions became gradually weaker after some days or weeks. Finally, the movement of the cilia on the gills also stopped, after which the cockle's body rapidly disintegrated. For this reason we took the movement of the cilia as a criterion of the cockle being either alive or dead. The average number of days that passed before the animals died is shown in table XIII as "fatal period".

As stated the cockles that had been kept for a time at —1.9°C did not all dig themselves in again. These evidently weakened animals did still make all sorts of movements with the aid of their foot; but this did not result in any directed displacement of the cockle, while these movements, too, became ever weaker in the course of a few weeks. Neither did the cockles keep their shell completely closed any longer, so that sand was often found within the mantle cavity. After this both the edge of the mantle and the gill filaments became slimy —they were found to have

been attacked by an enormous number of bacteria—, and soon afterwards the movements of the cilia also stopped. There were considerable differences in the individual fatal periods of the cockles; some already died after a few days; others after as long as a month and a half. All according as the temperature to which the animals had been exposed during the experiment had been lower and the period itself more prolonged, the death rate rose and the fatal period fell. After spending some time at a temperature of —2.4°C, a few animals were still alive; after a spell at —6.7°C and —15.9°C all cockles died. During these low temperatures, ice crystals formed on the tissues, giving the body a tough consistency. We assume that the lesions caused by these ice crystals are responsible in the first instance for the animal's death some days or weeks afterwards.

The above experiments show that cockles do not tolerate cold lower than about —1.8°C, i.e. the temperature at which sea-water with a Cl′ concentration of 18.5°/₀₀ freezes. But this applies only to cockles lying dry during a frosty period. The results were quite different when the cockles were first allowed to dig themselves into the sand, and then exposed to the lower temperatures. Then, cockles can stand even temperatures as low as for instance —7°C. In that case ice forms, both at the surface of the water and in the bottom where the cockle is living, but immediately above the bottom the water does not freeze; there, brine is formed which, at —7°C, proved to contain a Cl′ concentration of 66.2°/₀₀. Twenty-four hours afterwards the cockles were found to have suffered nothing whatsoever; they dug themselves in as rapidly as the control animals, and even six weeks afterwards showed no signs of any deterioration. It seems obvious that the explanation must be sought in the fact that during the experiment the cockles pump water of high salinity, so that the salinity in the tissues rises to a point where the formation of ice crystals is no longer possible; at any rate not to the same extent as in the "dry" experiments. No comparative analyses of the salinity in the cockles' tissues under the different conditions were, however, made.

We were able to test the results of the abovementioned experiments, made in 1948 and 1949, with observations during and after the cold period of February 1954.

Observations on February 4 and 6 showed that the high part of the flat (normal submersion time estimated at 4 hours per tide) was frozen down to a depth of 19 cm. At a depth of 10 cm, the temperature was —4°C. There was neither a layer of ice nor a layer of brine on the bottom. A number of cockles were hacked out of the bottom; they were naturally completely frozen; on breaking through the tough tissues one could see, with the naked eye, the glittering ice crystals. In view of the

results of our experiments we may assume that such cockles are no longer viable. Although, after thawing, these animals' gills still showed some movement of the cilia, this did not last long.

A little lower down on the flat (normal submersion time estimated at 5–9 hours) the ice sheet had an average thickness of 11 cm (minimum 8, maximum nearly 20 cm) everywhere. A long thermometer, stuck through a drill-hole into the bottom, showed that, at a depth of 15 cm in the bottom, the temperature varied between —2.5 and —5.9°C. Between the cover of ice and the frozen bottom there was a layer of brine with a Cl′ concentration of between 22.9°/₀₀ and 39.2°/₀₀. One may conclude from the bottom temperature of —5.9°C that this salinity must have been much higher still, at any rate temporarily; from data communicated by RINGER (1906) it can be calculated that the Cl-ion concentration must have been over 50°/₀₀. With a normal flood- and ebb-current, such abnormal concentrations in salinity would have been unthinkable; but during our presence on this flat we noticed nothing whatever that pointed to the existence of a tidal current under the ice. When the wind is east, the tidal currents across this fairly high flat are always weaker, because the average water level is then so much lower than normal. During the frosty period the wind practically always came from the East. The layer of ice on the flat, therefore, was raised only slightly by the flood, so that the water between the ice layer and the bottom remained almost stagnant. During the springtide of February 5, 1954, however, the flood-water was found to have forced its way, underneath the layer of ice, as far as beyond the normal high-water level on the flat.

About half-way between the high- and the low-water level we hacked a hole in the ice in order to collect a sample of cockles at this point. From the frozen bottom of the flat we extracted 13 cockles of 7 to 32 mm length. Their tissues, however, proved to be full of ice crystals, just as in the case of the cockles collected from higher up the flat; and the animals, which, in the laboratory, were slowly brought to a temperature just above freezing point, completely stopped moving their cilia after one or two days.

This contradicts the results of the abovementioned experiments, which led one to expect that the cockles would remain alive when, in frosty periods, a layer of brine has formed on the bottom of the flat. Maybe, however, these animals had already got frozen up before the formation of a layer of ice with its concominant layer of brine. Most striking, in this connexion, is an observation which we made after the thaw had set in, three weeks later. At a couple of hundred metres south of our sampling point there was a small, shallow creek, in which the cockles had actually remained alive, despite the fact that, at this point, the bottom was still frozen down to a depth of 10–15 cm. We assume

that a layer of ice with an accompanying layer of brine had formed there from the very beginning of the frosty period, and that it had prevented the formation of ice crystals in the cockles' tissues.

After two days of thaw, the ice-crust on the flat was still found to be a practically closed layer, while the bottom of the flat was still frozen down to 15 cm The layer of water between the ice-crust and the bottom no longer had a high salinity, however; the Cl′ concentration was below 10°/₀₀. This was surely due to the snow and ice being melted. This brackish water had a whitish colour, and smelt rather strongly of H_2S. Dead or half-dead Polychaets were lying all over the place. What we found in the way of cockles and other Lamellibranchs was dead. Even in the vicinity of the low-water level all cockles were dead. Whatever had not been killed by the frost must have died eventually, either through suffocation or by H_2S-poisoning.

As we just mentioned, we later found, just south of our traverse, a place where a number of cockles had maintained themselves. No doubt these animals too had been exposed to the frost; in this case we have to assume, first, that the brine below the ice prevented the formation of ice crystals in the cockles' tissues, and second, that due to local conditions the water at this point was not subsequently poisoned.

On another high tidal flat—the Zuidwal—we found in 1954 that, here too, the majority of cockles were dead. Nevertheless, a large number had survived in one or two places, despite the fact that these places are situated so high with respect to the low-water level that the frost, also here, must have penetrated deeply into the bottom. I am at a loss to explain how this is possible.

For our investigation into the cockle's year-class 1947, therefore, it was an exceptionally favourable circumstance that there were no periods of severe frost between 1947 and 1954. During the periods of moderate frost of February 1948 and March 1949, the bottom of the flats did not get frozen up, although a layer of ice formed for a short time over the higher parts of the flat; this layer of ice, however, was nowhere a continuous mass, and at each tide the relatively warm flood-water reached the normal high-water level of the flat. These tidal currents admittedly did bring along some ice-drift, which—as we observed also elsewhere—may be injurious to the cockle population. But mortality was never serious. On February 28, 1948 we noticed that, on the Zuidwal, large numbers of cockles near an ice barrier were washed up and transported to deeper water, where, as we know, they have no chance of survival. This, however, was merely a local phenomenon; and we accordingly do not suspect that the mortality in the period from 1947 to 1954 has been influenced by the frost to any considerable extent.

If we then inquire to what extent *high* temperatures may have some effect upon the mortality, the following experiments will give some insight into the degree of resistance of the cockles against these high temperatures (see table XIV). These experiments were made, in the summer of 1949, with animals that had been collected on the flat shortly before. During the experiment the temperature of the water was raised in the course of a few hours to a certain point (= maximum temperature) and maintained at this point for a period of 1 to 24 hours. After this the temperature was gradually lowered to a normal value in the course of a few hours. At the end of the experiment the mortality among the test-animals was recorded for a period of one week.

TABLE XIV

Mortality of cockles after a temporary raising of the temperature

average length of the cockles in mm	(min-max. length in mm)	numbers of animals	initial temp. in °C	max. temp. in °C	duration of max. temp. in h.	mortality after 1 week numbers of of animals	per cent.
4.4	(3–6)	25	18	31	24	0	0
29.5	(26–35)	25	18	31	24	0	0
4.6	(3–6)	25	18	34	0.1	1	4
28.9	(25–33)	25	18	34	0.1	2	8
4.5	(3–6)	25	19	34	1	3	12
29.0	(26–35)	25	19	34	1	5	20
4.6	(3–6)	25	18	34	12	18	72
29.4	(26–35)	25	19	34	12	24	96
4.4	(3–6)	25	20	36	0.1	25	100
28.8	(25–34)	16	20	36	0.1	16	100

As table XIV shows, temperatures of 31 °C are tolerated for 24 hours. But at 34 °C some mortality sets in; after the cockles have been exposed to 34 °C for 6 minutes, a small proportion of them already die. A longer stay in water of 34 °C raises the mortality. The spat (3–6 mm) appears to have greater resistance than the two-year-old cockles of 25–35 mm. Not a single cockle, however, can stand a temperature of 36 °C for even 6 minutes.

We do not suppose, however, that the temperature within the bottom of the Dutch sand-flats can ever rise to above 31 °C. What we did find was that where, at low water, small puddles are formed with a water level of a couple of centimetres, it is possible for the temperature of the water to rise to above 30 °C. At a water temperature of 32 °C, the bottom, at a depth of 1 cm, was found to have a temperature of 29 °C.

If the cockles are capable of stopping their respiratory current during

the few hours that such high water temperatures continue they will not suffer any injury. But if they are *not* capable of this, they get the warm water into them direct, together with the possibility of mortality setting in. Since, however, the area covered by these shallow puddles has hardly any significance in the field we investigated, any possible mortality in these puddles cannot have much influence on the total picture of the mortality on a tidal flat.

On every flat, of course, the temperature of the bottom in the summer may rise, during low-tide, to above 20°C; but from the moment the flat emerges the consequent evaporation will be so strong that the temperature—at any rate on a sand-flat—will fall again. An example of this is given in the following table, drawn up when the bottom temperature was temporarily even higher than that of the air.

TABLE XV

Course of the temperature on the Zeehondenplaat just above the bottom, and 1 cm deep in the bottom. July 2, 1951

2-7-1951	water level in cm	temperature of the air (°C)	water temperature (°C)	bottom temperature (°C)
13h.10	21	18.7	17.3	17.2
13h.20	13	19.3	19.0	19.0
13h.26	10	19.0	19.7	19.6
13h.35	5	18.4	21.1	21.0
13h.39	3	18.9	23.2	23.4
13h.43	0	18.9	—	22.8
13h.55	dry	19.7	—	17.1

Since we do not assume that temperatures above 31°C can ever occur in a sloping flat-bottom with reasonably good drainage, we do not expect that high temperatures can be a main cause of mortality among cockles. None the less, we sometimes found that many older cockles died in summer during a very hot period. Thus, in the beginning of July 1952, we found locally on the Zeehondenplaat a big proportion of the year-class 1947 with their shells open. This gaping is a sure sign that the fatal process has started. And in fact, 2 weeks later we found no more live cockles of the 1947 year-class, although the one- and two-year-old cockles had maintained themselves perfectly. I therefore assume that the primary cause of death was not the high temperature, but the fact that the old cockles had already been weakened, either by parasites, or as a result of senility.

It is furthermore remarkable that, each year in April or May, during a period of relatively warm weather, many cockles may be found on the surface of the flat with their shells partly open. ORTON (1932) suggests as a possibility that such cockles may have been weaken-

ed by the production of eggs and sperm, and may be unable to tolerate a *rapid* rise in the temperature. The same author (1934, p. 117) pointed in this connexion to the extreme differences in temperature between the flat which is warmed by the sun, and the very cold flood-water that will presently cover the flat again. A number of experiments which we made in the second half of April 1949, in which two-year-old cockles were transferred from water of 20–25°C to water of slightly over 0°C, and returned, 6 hours later, to water of 20–25°C, failed to provide any evidence that cockles take badly to such sharp fluctuations in temperature. For the first week after the experiment there was not a single dead cockle among the 25 specimens so tested.

For that matter, it is precisely in April and May that cockles show their fastest growth (see p. 416); and it is therefore doubtful whether they pass through a period of weakness during those months. As, however, our experiments have shown that, in the cold season, the fatal process may take several weeks, we suspect that the springtime mortality involves those animals which have already been weakened during the preceding winter. In that case it may be possible for the fatal process to be accelerated by sharp fluctuations in temperature. We shall revert further on to the part played by parasites in this process.

Summarizing we may say that, so far as the temperature is concerned, it is especially the low temperatures that may cause excessive mortality. This mortality may be the result of direct freezing, when ice crystals form in the tissues, but it may also be due to suffocation, i.e. when there is a stagnant layer of water between the bottom and the layer of ice on the flat. We do not believe that either high, or sharply fluctuating, temperatures are of any significant influence on the mortality in the area investigated.

4. *Influence of light*

A factor whose influence on the mortality of cockles is not yet clear to us, is light. As we remarked before, cockles are found only rarely, in the Wadden Sea, below a depth of 2 metres, although a large quantity of spat must surely come down there. In the Westerscheldt Estuary on the other hand, large numbers of cockles are found at a depth down to 4–5 metres below low-water (F. Duringk, Supervisor of Fisheries, Hansweert; oral communication). In the North Sea we found a cockle bank at 14 m, while, according to the local fishermen, cockles are even found there at a depth of 20 metres. Now, the North Sea water is relatively clear; close to the place where we found the cockle bank, a Secchi disc was still visible at a depth of over 9 metres below the surface. In the

Wadden Sea this disc already disappears from view, in most cases, at a depth of ca. 3 metres. The Westerscheldt water—according to our impression—is about half-way, as regards turbidity, between the North Sea and the Wadden Sea.

VERWEY (1952, p. 175) defends the view that the cockle's need for light is a determinant of the depth at which the animals are found. According to this argument, the quantity of light would be about the same at a depth of 15–20 metres in the North Sea and 3 metres in the Wadden Sea, an assumption which, in view of the difference in turbidity, could be quite reasonable. Moreover, cockles are extremely photosensitive. Their photokinetic sense-organs are located on the tentacles of the respiratory siphons; i.e. on those parts which normally protrude beyond the shell (JOHNSTONE 1899, p. 60). When a shadow falls across the siphons, the cockle at once draws them in and closes his shell. This reaction also takes place upon the approach of a fish—even before the fish's shadow has reached the cockle, as we observed in our laboratory; but when it is dark, of course, this reaction is eliminated. Now we have repeatedly found young flatfish (plaice and sole) whose stomach contents consisted chiefly of bitten-off siphons of *Mya* spat. Admittedly, the siphons of *Cardium* are so much shorter than *Mya's* that it may perhaps be impossible for a flatfish even to bite off a *Cardium* siphon; but we may nevertheless assume that the cockle, by quickly closing up its shell on the approach of a fish, effectively escapes the attack of a potential predator. BRAUN (1954) succeeded in demonstrating, with respect to *Cardium oblongum*, that the reaction in question occurs in a slower tempo in a darker environment. If this should apply also to *Cardium edule*, it appears probable that, below a given limit of light intensity, a cockle population will rapidly fall a prey to flatfish and similar enemies.

According to SMIDT (1951, pp. 83 and 116), conditions for the cockle's growth are not unfavourable in the deeper water of the Wadden Sea, only the flatfish preventing a cockle population from maintaining itself there.

If we should base our argument on a possibly existing influence of light, then it would follow that the flatfish is less troubled, in catching its prey, by a lower light intensity, than the cockle is in parrying the attack. It is true that nothing is so far known upon this point; but it appears far from improbable. None the less, the absence of cockle banks in the deeper water of the Wadden Sea cannot be completely explained by the presence of flatfish, since there are very large numbers of flatfish along the coastal strip in the North Sea where the cockle bank, referred to above, was found.

Furthermore, one may imagine that the light could affect the cockle's

behaviour in yet another way, namely with regard to the speed with which a cockle digs itself in after having been washed out. If a cockle should dig itself in either less quickly or less deep in the dark than in the light, then this, too, might be the cause of the animal's falling a prey to flatfish or other predators, at a low light intensity. In May, 1953 we made a few experiments in which the digging-in speed of cockles was determined in the light and the dark respectively. 20 One-year-old cockles (average 15.3 mm length) and 15 two-year-old ones (25.3 mm) were placed alternately in a light and in a dark aquarium. Of each cockle the time was noted that passed before the specimen in question put its foot out ("latency period"); and after this, the time that passed before the animal was completely dug in. In the experiments made in the dark, the position was recorded minute by minute with the aid of a pocket torch. Cockles with a latency time of more than 30 minutes were left out of consideration. Only one or two cockles in each experiment fell in this class. The results are found summarized in table XVI.

TABLE XVI

Digging-in time of cockles in light and in darkness. Water temperature 12.4°C

animals'age	total number	latency period (min.-max.)	digging-in time (min.-max.)
		in the light	
1 year	56 specimens	7.2 min. (1–30 min.)	5.5 min. (3–14 min.)
2 years	42 specimens	14.3 min. (5–27 min.)	19.3 min. (12–41 min.)
		in the dark	
1 year	56 specimens	8.6 min. (1–28 min.)	6.7 min. (4–27 min.)
2 years	43 specimens	14.8 min. (4–26 min.)	16.8 min. (13–29 min.)

As is evident from the above data, there is no great difference in the rapidity whith which a cockle digs itself in, as between light and dark. Small cockles work themselves under the sand more quickly than large ones—as we also saw on p. 377; but there is practically no evidence whatever of any influence on the part of the prevailing light.

We would finally mention the possibility that the light intensity might have a more direct effect on the mortality of the cockles. We suggest this possibility on the ground of the following observation.

In three pairs of experiments relating to the growth of young cockles it was found that, in the three experiments made in the dark, the mortality was slightly higher than in the three experiments made in the light. These experiments comprised a total of 2600 small cockles of from 2 to ca. 6 mm, all of which were kept from August 3rd until Sep-

tember 21st 1954 in large concrete tanks, together with a certain concentration of green Flagellata that were to serve as their food. The oxygen content was maintained at practically saturation point with the aid of air bubbles. In the course of these 7 weeks the mortality of these small cockles that were kept in the dark was as high as 80.5 per cent.; of those kept in the light, on the contrary, only 44 per cent. Since, however, both in the light and in the dark, the growth of the cockles during this period was only about half the normal growth on the tidal flat, it is certain that the general conditions prevailing in our experiments were insufficient, so that no great value must be attached to the high mortality in the dark; in fact, the experiments in question constitute no more than an indication that the mortality might perhaps be affected also directly by the light intensity.

Admittedly, the experiments of Mr. C. Bas (Verwey 1955, p. 144) might be taken to imply that darkness has no influence on the mortality of one-year-old cockles; but in his experiments, too, the general conditions were such as to preclude any normal growth altogether.

There is another point in which the course of the mortality during our experiments differs from the mortality of the cockles we found in the deeper parts of the Wadden Sea. During the 7 weeks that our experiments lasted we never found more than a few dying cockles at one and the same time. In the Wadden Sea, on the contrary, we found to our surprise that the few times we discovered cockles in deeper water, there were invariably, between the living cockles, many that were either dying or only just dead. Evidently, therefore, this mortality took place at short term, and certainly not in the course of a long period. Equally certainly, the cockles which we obtained from the Wadden Sea channels had not suffered from prolonged exhaustion; for their weight was perfectly normal. This we conclude from a lot of cockles which we collected in November 1953 in the Oude Vlie, just east of the Zeehondenplaat, at a depth of $2^1/_2$ metres. A proportion of these cockles had only just died—the dead bodies inside the shells were still unaffected. Another proportion were still healthy; at any rate they reacted normally when touched. But by far the greater majority were in rather poor condition—when touched they either closed their shells only very slowly or not at all. There was no doubt that they were in a dying condition. Their weight (expressed in dry weight without the shell), however, was higher, rather than lower, than that of the healthy cockles (table XVII); their death did not, therefore, involve a long process, while it is also evident that they had not fallen a victim to predatory animals.

It looks as if these cockles had originated from the Zeehondenplaat, and been transported, either in a healthy or in a sick state, to the channel.

TABLE XVII

Dry weight of dying and of healthy cockles. Oude Vlie, at 2 ½ metres' depth. 3rd November 1953

	average length	numbers of specimens	average dry weight of soft tissues
dying cockles	29.1 mm	64	0.374 gr.
healthy cockles	29.0 mm	82	0.365 gr.

The bottom which they found there consisted of fine, muddy sand rich in H_2S, covered by a layer of detritus. There is no doubt that this substratum offered the cockles exceptionally unfavourable living conditions, in which the light can surely not have played a prominent part. A more obvious explanation seems to be either lack of oxygen or H_2S-poisoning.

Although it would be wrong to draw too far-reaching conclusions from this single example, it does at any rate show that lack of light alone should never be held responsible for the absence of cockle populations from the deeper parts of the Wadden Sea. In the quiet, sheltered parts, where the cockles that have been washed away are bound to land in many cases, the soft muddy bottom, rich in H_2S, probably does not offer suitable living conditions.

Our conclusion is that there are admittedly some indications that the light may affect the mortality chances, but that no definite proof of this can as yet be given. In any case it seems out of the question that the high mortality in the deeper parts of the Wadden Sea should be *chiefly* due, either directly or indirectly, to deficiency of light.

5. *Influence of the available quantity of oxygen*

That the cockle mortality could be affected by the amount of available oxygen appears to be precluded. The water on the flats is almost constantly saturated, or even oversaturated; and no evidence has ever been produced of the existence of seasonal fluctuations in the oxygen content of the water. If the spat has succeeded in settling there is no reason to assume that cockles at a later age should suffer from a lack of oxygen—apart from exceptional cases. We have already referred to one of such cases, when discussing the mortality during frosty weather; in this case there may occur deficiency of oxygen underneath the layer of ice that is formed on the flat. It might also be possible that cockles washed away from a flat land in a creek at a sheltered, muddy spot, and perish there as a result of oxygen deficiency (or through H_2S poisoning), as suggested above.

6. *Influence of the structure of the substratum*

As we have already noted, the mortality on the Zeehondenplaat is higher in the creek than on the tidal flat, while the creek is characterized by a soft muddy bottom (fig. 4, p.362). We shall therefore inquire into the possibility that the mortality among the cockles might be higher on flats with fine sand, rich in mud, than on flats with coarse sand and without mud. Table XVIII contains the available relevant data.

TABLE XVIII

Mortality and bottom-structure on the flats in the years 1948–1949

	percentage of annual mortality	average grain size in μ	mud in gr/l.
Creek Zeehondenplaat	68.5	78	126
East part of Zuidwal	47.7	133	4
Flat Zeehondenplaat	43.5	113	60
South part of Staart van Schieringhals	41.6	95	16
West part of Zuidwal	30.1	105	58
Paardenhoek	27.0	122	15
North part of Staart van Schieringhals	10.2	104	11

From the above figures we may conclude that the grain size of the sand has evidently no influence on the mortality. Neither is there any noticeable connexion between the relative quantity of mud and the mortality; it is only remarkable that it is precisely the creek of the Zeehondenplaat, with its high mortality, that is so extraordinarily rich in mud. Unfortunately we do not possess any comparable data relating to other flats exceptionally rich in mud, although we have the impression that there, too, the mortality will be abnormally high. Thus, we saw (in 1951), on the extremely muddy parts of the Zuidwal, south of the Vangdam, a dense population of spat (300–700 specimens per 0.2 m^2) disappearing completely in the course of the winter, whereas, on the sandy parts close by, the population of about 40 specimens only decreased to over 20 per 0.2 m^2. We made a similar observation on 1952, and again in 1956, on a small flat along the harbour of Den Helder. Further, we were repeatedly struck with the fact that on the very muddy flats along the coast of Friesland and the Lauwerszee nothing but spat and a few one-year-old cockles were found, while, on the sandy parts, there were also large numbers of older animals.

While, therefore, there usually is a considerable spat-fall in muddy areas, it appears that the mortality, too, is high in these places. The direct cause of this high mortality cannot possibly be ascribed to the danger that cockles, as they get heavier during growth, would sink down into the mud. In the first place, these mud-flat bottoms are not

so very soft that a cockle would merely sink down into the mud from its own weight; for the cockle's specific weight is, after all, lower than that of the mud; but apart from this, on these muddy flats the empty shells are not found in the bottom *there*, but elsewhere.

We have also asked ourselves whether it might not be the reduced state of the muddy bottom that could be injurious to the cockle. But on repeated occasions we found cockles in very much reduced mud—both on the Zuidwal and on the Balgzand—, and the shells of the live specimens so discoloured as to be completely black, without this having apparently affected either their growth or the population density. None the less, it is far from unthinkable that, under certain conditions, a reduced muddy bottom might definitely be harmful to the animals. We hope to get a clearer insight into this aspect by further experimental investigation.

7. *Influence of detritus*

We do not possess many factual data relating to the quantity of detritus which is carried across the flat-bottom along with the current. We repeatedly found, along the channels, and below low-water level, large quantities of peat-flakes, fibres, alga remnants, etc., just at those places where one would expect to find cockles. However, they were conspicuous by their absence. Furthermore, as we saw before (fig. 4, p. 362), much of this detritus was deposited in the creek on the Zeehondenplaat, i.e. where the cockle mortality was highest. Nevertheless, we find it difficult to assume the presence of any direct influence of suspended detritus on the cockle's expectation of life. We shall return further on to the question of a possible influence of this detritus or silt on the cockle's rate of growth.

Finally, there is the possibility that detritus suspended in the water may affect the mortality. As we shall see later on (p. 434) it is possible that large quantities of suspended detritus—such as are found along the coast of the provinces of Friesland and Groningen—adversely affect the cockles' growth. But since the cockles which we found there were only rarely older than 2–3 years, it does not appear unthinkable that this detritus in the water affects, in addition, the animals' duration of life.

8. *Influence of biotic factors*

As biotic factors which might influence the mortality rate, we would mention, in order of sequence: food, competition for space, predatory animals, and parasites.

There is no doubt whatever that the available quantity of food may affect the mortality. As the investigations of Thamdrup (1935), Kreger

(1940) and SMIDT (1950) have already shown, the rate of growth of the cockle is dependent upon the available quantity of food. An unduly small amount of food—for instance as a result of a very short submersion time—will surely be fatal. As the food factor is primarily important for the cockle's growth, this question will be discussed in more detail under the latter heading. We shall content ourselves at this point with stating that we have no indications showing that lack of food, except in zones of too short submersion time, is, as such, a cause of death (p. 367).

Competition for space

KREGER (1940, p. 182–184) describes how the density of a cockle population may be so great that the animals press one another out of the bottom. This happened in a population whose density was 2169 specimens per m^2, at an average length of ca. 25 mm per animal. In the banks we investigated we never found a population of such density that it would be possible for the animals to crowd one another out.

Neither did mortality resulting from overlaying by mussels, as described by KREGER (1940, p. 191), occur in the areas investigated by us in the period from 1947 to 1956, although this factor did appear to be of influence elsewhere in the area under observation. Wherever a mussel bank establishes itself, the cockle population is doomed to death; but the total area covered by mussel banks is quite small—it can be estimated at about 1 per cent. of the Wadden Sea area—, while that occupied by cockles should be, on a rough estimate, about 50 per cent.

Predators

As the course of the mortality curve in fig. 7 (p. 371) shows, the *absolute* mortality of the year-class 1947 is very high during the first few years; but it steadily decreases during subsequent years—at any rate until halfway down the summer of 1952. This shows that the cockles do not die of physiological senility, for in that case one would rather expect an initially slow, and subsequently rapid decrease of the population. It most resembles a process in which the mortality is determined in considerable measure by predators. But what animals prey upon cockles? Locally, at any rate, plaice *(Pleuronectes platessa)* is surely capable of devouring a large number of young cockles, as REDEKE (1906, p. 98) and SMIDT (1951, p. 115–118) demonstrated. The flounder *(Pleuronectes flesus)* is able to use small cockles as the main component of his menu—at any rate in a non-emerging area such as the former Zuyderzee (VAN BREEMEN and REDEKE 1907, p. 12). On the flats in the tidal zone the flounder appears occasionally to eat cockle spat (REDEKE 1906, p. 103; BLEGVAD 1917, List 6), but no adult cockles. On the tidal flat the

flounder has no significance as a consumer of cockles, as is evident from SMIDT's data (1915, p. 114), and as is also illustrated by our own results: the stomach content of 20 flounder (14–28 cm) which were caught on the Zeehondenplaat in July 1950, at a falling water-level, when they left the flat to seek deeper water, amounted altogether to 18 *Gobius minutus*, 135 *Crangon crangon*, 37 Mysidacea, 12 Amphipoda, and 12 Polychaeta, but not a single mollusc.

Concerning the part played by the shore-crab *(Carcinus maenas)* in exterminating cockles as ORTON (1926, p. 243) describes from his experiments in the neighbourhood of Plymouth, we have no observations.

Maybe the herring-gull, or the enormous numbers of oyster-catchers which, during migration time, collect around the isle of Griend, could be held responsible for the disappearance—already referred to—of the local cockle population in the autumn or winter of 1947–1948 and 1948–1949. It is certain that migratory birds must have levied their toll particularly on the emerging part of this tidal flat; but the fact that, along the Blauwe Slenk, cockles did survive, and not in the initially so densely populated creek just south of Griend, points to the possibility that, at this point, other factors may also have been of influence on the situation.

Parasites

The extent to which the mortality among cockles is caused by parasites has hardly been, so far, a subject of investigation. It is quite possible that parasites are an important factor of death, but no sufficient data are available.

It is our impression that the presence of only a few old cockles per m^2 may considerably increase the mortality of the growing spat. As we mentioned before, a dense population may obstruct the settlement of spat (p. 368); but we here refer to something different: in the years following 1947 we repeatedly observed that, even when the spat-fall was not too small, the spat in question disappeared completely in the course of one year, also in places harbouring only a few older cockles of the year-class 1947. Not until the total disappearance of these older cockles did a new year-class establish itself definitely. This we noticed especially on the Zeehondenplaat, the Zuidwal, and the Balgzand (in spite of the fact that KREGER (1940) did find a mixed population on the same flats in the years from 1935 to 1939; he too, however, mentions the predominance of one particular year-class). THAMDRUP (1935, p. 137) mentions the same phenomenon in the Danish Wadden Sea, and puts forward as his impression that, in the presence of more than 100 adults per m^2, even in favourable conditions, the spat has small chance of surviving the first winter. SMIDT (1944, p. 18–19) confirms this, and

wonders whether its cause should be sought in food-competition alone, or in addition to some form of infection that might attack the young cockles. If we are right in assuming that the mere presence of a few older cockles raises the mortality of the younger ones, there is, in fact, good reason to suggest that some form of infection might endanger the young cockles' life. A limited investigation instituted on the Zeehondenplaat in 1955, into the possible nature of such an infection did not, however, yield any positive result; in the spat of 10–15 mm length we did not find many parasites, although the mortality was considerable.

Furthermore, it does not appear impossible that parasites do play some part in causing the cockle mortality in springtime. In the early spring of 1948, and in May 1949, we found, in the body-liquor of gaping (i.e. dying) cockles, a species of Flagellata in large numbers. These were not identified; but in view of their location it is possible that they may have been some form of *Hexamita*. *Hexamita* is known as the cause of the "pit-disease" in oysters (Mackin and co-workers 1952). The gaping cockles' intestine, moreover, contained a large number of Gregarina, while the gill-tissue was full of bacteria. It is not clear to what extent these organisms were either the primary cause or only an accompanying factor in the somewhat drawn-out mortal process in these gaping cockles—the more so because no healthy animals were examined at the time.

In 1950, Mr. J. H. Stock (Amsterdam) called our attention to a parasitic copepode, *Paranthessius rostratus*, which lives in the mantle cavity of both large and small cockles. Ever since 1951 we have been finding this copepode—particularly in summertime—in large cockles; in some cases there were some tens of them in a single cockle. It is not known, however, to what extent this copepode causes serious trouble to his host. Moreover, Orton (1934, p. 102) states that, in the spring mortality along the west coast of England, he did not find, at any rate during a perfunctory investigation, the presence of any *Paranthessius* specimens.

Smidt (1944) mentions the possibility that infection by Trematodes might entail a considerable degree of mortality. For he found that an entire population may be attacked by these parasites, but he has no observations concerning the results of this infection. In the Western Wadden Sea we admittedly never came across an infection by Trematodes; but our observations on this point are only limited to a few.

Although the total number of observations relating to the mortality of cockles is not so very small, they still are insufficient for a solution of the problem of how the local differences in mortality on a tidal flat such as the Zeehondenplaat are to be explained. True, the fact that, where

conditions for growth are bad the mortality is relatively high, does provide some indications—which will be discussed further on, under the heading "growth"; but in addition to this a closer investigation into the part played by predators and parasites will be required in the future.

IV. LOCAL DIFFERENCES IN GROWTH

The brief, but abundant spat-fall in the early summer of 1947 was exceptionally favourable for studying the growth of the cockle under different milieu conditions. In the course of the investigation it was found that, in some cases, it makes a difference whether the length of the shell is taken as the criterion of the cockle's growth, or some other measurement. In deciding upon the measurement to be chosen as the criterion of growth we have the choice between the length, the height, the thickness and the volume of the shell; but we may also choose the animal's weight—either that of the shell, or that of the flesh. Finally, we made a small number of observations concerning the possible significance of differences in the number of diverging ribs in relation to differences in growth.

In our investigation we almost invariably took the increase of the length of the shell as a measure of growth. The measure of length has many practical advantages: its determination is extremely simple, and in fact, this measurement has always been used by earlier investigators as well, so that our results can be easily compared with theirs. But the greatest advantage of the measure of length is that the values obtained can at once be compared with the values of the winter-rings, of which only the length can be measured. The difference between the total length of the cockle and that of the winter-rings is a direct measure of the animal's growth during previous periods.

Before proceeding to discuss the factors causing the local differences in size we shall first try to ascertain whether such differences in size are a result of a *lasting* difference in the relative rate of growth, or a result of the perpetuation of a difference in length which, for instance, might already have arisen during the animals' first few months of life.

In order to understand the nature of the local differences in growth we determined, on the Zeehondenplaat, each year from 1947 to 1953, the growth of the different year-classes of cockles, in which naturally the especially rich year-class 1947 predominated.

A rough idea of growth of the cockles on the Zeehondenplaat can be obtained from fig. 10 (p. 375), in which the frequency distribution of the length of the cockles from a given spot is represented by black squares, and the frequency distribution of the first winter-ring of the same specimens by

a corresponding blank square. While the frequency distribution of the length distinctly shifts upwards during successive years, the frequency distribution of the first winter-ring remains approximately the same, and corresponds to that of the length of the cockles in the first year (1947). As we pointed out before, this may be taken as evidence that the cockles moved about very little—at any rate before 1950—; from this it further follows that we can consider the change in length in a given locality as synonymous with growth.

The cockles from the samples situated on the top of the flat (i.e. Nos. 10–38, of which only Nos. 10–16 are included in fig. 10) possess a large first winter-ring. But the average length of this winter-ring differs considerably as between one sample and another, and as between one year and the next. In view of the small number of cockles per sample, and the great individual differences in growth, we believe that the differences between the samples on the top of the flat are the result of accidental differences in sampling, and are not, therefore, significant. We considered it preferable, therefore, to determine these cockles' average growth, since thereby accidental differences are largely neutralized.

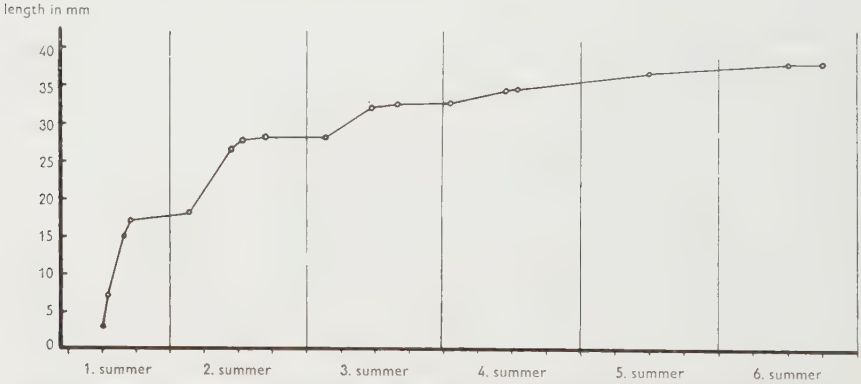

Fig. 11. Curve construed to show the growth of cockles on the top of the Zeehondenplaat, from data of three different year-classes (1947, 1951 and 1952).

Fig. 11 represents the growth on the top of the flat, determined in this manner. Not all the data utilized here derive from one and the same year-class; in order to get as detailed an idea of the animals' growth as possible, we used the data obtained from 3 year-classes. For the first summer the growth of year-class 1952 was taken; for the second summer, that of year-class 1951, and for the other periods, that of year-class 1947. This "construction" of the growth is permitted because the lengths of the winter-rings for the year-classes 1947, 1951 and 1952 show sufficient similarity to justify the conclusion that the growth proceeded on the same lines in all three year-classes.

Looking at the details of the course of the growth on the flat, we shall note that the increase in length chiefly takes place in the spring and early summer. This is naturally most distinctly noticeable in the animals' first years of life, when the increase in length is considerable; but it also applies to older cockles. Following a stagnation in growth during the winter, the length increases rapidly from March—or, in a cold spring, from April. In the course of July the rate of growth decreases again, and after August the cockle hardly grows any more. This phenomenon was not only found on the Zeehondenplaat; the data from other flats investigated all point in the same direction. But the same does not apply to the *spat;* the spat continues to grow considerably until the end of September—a circumstance to which we shall revert later on (p. 418) In our opinion this is an indication that the short duration of the almost explosive growth after the winter stagnation is not only due to the conditions for growth prevailing in the environment, but must also be a result of the internal regulation of the cockle.

Let us now look at the rate of growth on the different parts of the Zeehondenplaat. As already stated, the cockles of the samples from nearer the creek (i.e. Nos. 1–9 in fig. 10) have a smaller average winter-ring than those on the flat itself. For the creek, we took the four deepest sampling spots as being representative (Nos. 3–6). As the year-classes 1951 and 1952 in the creek show a slightly stronger growth than the year-class 1947, it was not possible to construe, for the cockles from the creek, as detailed a growth-curve as for the samples from the flat. For the creek, therefore, we can only give the annual growth of the cockles of year-class 1947. For the creek, moreover, we have to take into account the importation of cockles from the flat in the years after 1949 (see p. 376); for this reason, fig. 12 shows the growth from 1947–1949 only, and the growth of the creek-cockles after 1949 will accordingly be left out of consideration.

Now examing the growth-curves of the cockles from the flat, and of those from the creek (fig. 12, p. 404), we shall see at once that the absolute increase in length steadily diminishes in the course of the years. What is remarkable, however, is that the cockles keep on growing until they die—at any rate until their eighth year of life. In this respect, therefore, their development shows no definite closure.

The course of the growth-curve at once precludes the possibility that the cockle should increase in size by a process of continuous "compound interest". For, in that case the absolute increase in length per year would be greater instead of smaller in the course of the animal's development. Not only the absolute, but also the relative, increase in length diminishes in the course of the animal's life.

From the regular shape of the growth-curve it seems likely that the

increase in length, during the period in question, is a simple power-function of time. If this assumption should be right, then the points in fig. 12 would come to lie in a straight line, when both axes in fig. 12 are given a logarithmic scale. As fig. 12a shows, these points, in

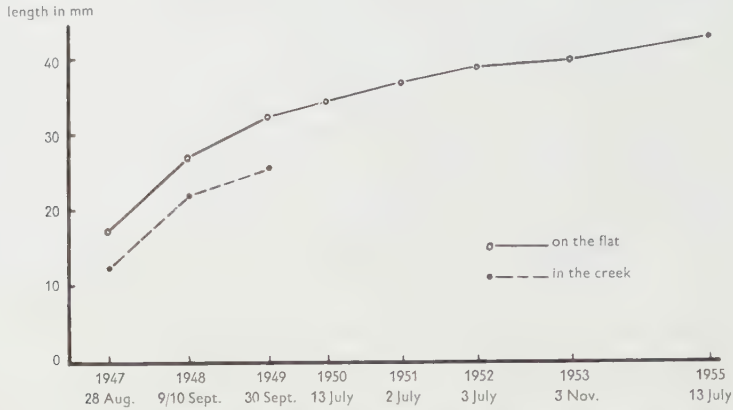

Fig. 12. Growth of the cockle year-class 1947 on the flat and in the creek of the Zeehondenplaat.

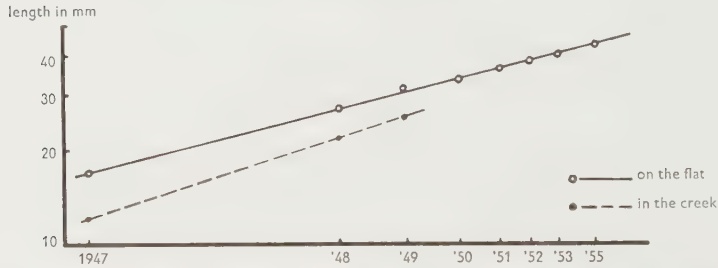

Fig. 12a. The same data as in fig. 12, but both axes of the figure with logarithmic scale.

fact, do lie practically in a straight line; i.e. a line of which the co-tangent of the angle which it forms with the abscissa, indicates the power with which the length approximately increases. This, of course, applies only to the distance over which the line is straight. We then find that the length of the fast-growing cockles on the flat increases by the 4.1-power root of the time, and that the length of the slower-growing cockles in the creek increases by the 3.5-power root of the time. This implies that not only the absolute increase in *length*, but also the relative increase in *volume* per year, both diminish in the course of the animals' life—and more so in the case of cockles on the flat than in those in the creek.

TABLE XIX

Annual winter-length and percentage-increase in length of cockles on the top of the flat and in the creek of the Zeehondenplaat

year-class 1947, Zeehondenplaat	length 1st winter-ring in mm	length 2nd winter-ring in mm	length 3rd winter-ring in mm	rate of growth between 1st and 2nd ring (No. of specimens)	rate of growth between 2nd and 3rd ring (No. of specimens)
Flat-cockles	19.4	28.1	32.0	44.7 per cent. (664)	13.9 per cent. (198)
Creek-cockles	14.0	20.6	25.6	47.1 per cent. (195)	24.3 per cent. (172)

This latter point might not, perhaps, have been expected; but the annual relative rate of growth of the flat-cockles is slightly lower, both in the second and in the third year of life, than that of the creek-cockles (see table XIX).

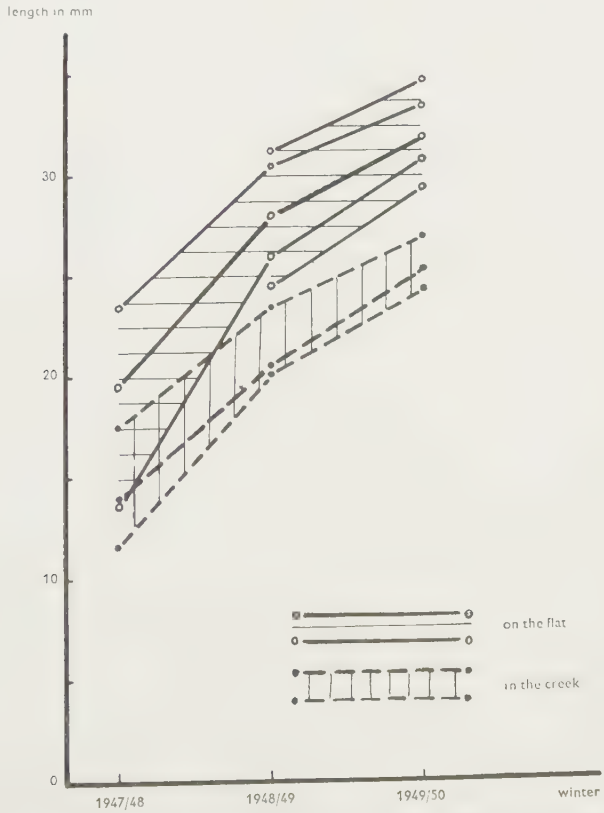

Fig. 13. Increase in length, between the first and the third winter, of large, medium-size, and small cockles of the year-class 1947, from both the flat and the creek of the Zeehondenplaat.

TABLE XX

Annual winter-length and percentual growth of the various length-categories of the cockles on the flat and in the creek of the Zeehondenplaat (Numbers of cockles are printed in parentheses)

Zeehondenplaat year-class	length of 1st winter-ring in mm	length of 2nd winter-ring in mm	length of 3rd winter-ring in mm	percentual growth between 1st and 2nd winter-rings	percentual growth between 2nd and 3rd winter-rings
Flat-cockles 1947					
1st ring 10– 15 mm	13.6	26.0	30.8	90.4 (40)	18.5 (26)
1st ring 16–22 mm	19.1	27.8	31.9	45.6 (529)	14.7 (136)
1st ring 23–28 mm	23.5	30.6	33.4	30.2 (95)	9.2 (36)
2nd ring < 26 mm	—	24.4	30.2	—	23.9 (22)
2nd ring > 29 mm	—	31.2	34.8	—	11.5 (15)
Creek-cockles 1947					
1st ring 9–12 mm	11.8	20.2	24.8	74.1 (48)	22.8 (43)
1st ring 13–16 mm	14.1	20.0	—	41.9 (117)	— (0)
1st ring 17–20 mm	17.6	23.4	27.1	33.0 (30)	15.7 (6)
Flat-cockles 1951					
1st ring 5–11 mm	6.9	25.6	—	271. (11)	— (0)
1st ring 14–16 mm	15.3	26.9	—	75.2 (20)	— (0)
1st ring 17–19 mm	18.0	29.4	—	63.4 (93)	— (0)
1st ring 20–22 mm	20.2	29.9	—	48.1 (22)	— (0)
Creek-cockles 1951					
1st ring 7–12 mm	11.3	22.2	—	96.5 (445)	— (0)
1st ring 13–16 mm	13.7	23.8	—	73.7 (1035)	— (0)
1st ring 17–20 mm	17.5	26.2	—	49.7 (45)	— (0)

From the fact that the first winter-ring of the flat-cockles is so much larger than of the cockles from the creek, it may be concluded that, in the first summer of the animals' life, not only the absolute, but also the relative rate of growth on the flat exceeded that in the creek; but after the first winter, the relative rate of growth on the flat falls below that in the creek. When, however, we then compare the growth of animals of equal initial size, we shall find the rate of growth on the flat, also after the first year, to be considerably in excess of that in the creek. This is clear from table xx, in which the cockles from the flat and from the creek, respectively, are divided into three categories according to length; the larger, the medium sized and the smaller individuals from the flat and the creek having been determined separately. Now on comparing the flat- and the creek-cockles with equally long first winter-rings, the animals on the flat will be found to have grown much faster, the following year, than those in the creek (see fig. 13). The same applies to the

second year, and doubtless also to succeeding years. That, therefore, the mean relative growth on the flat is less than in the creek, is entirely due to the fact that the flat-cockles have reached so much greater a length at a much earlier time.

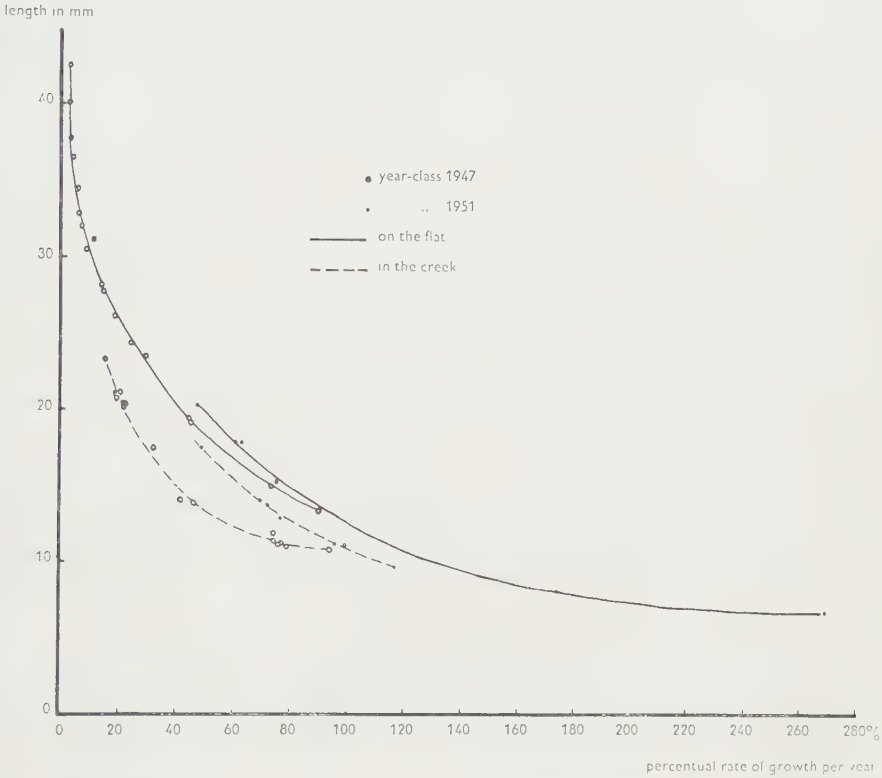

Fig. 14. Percentual rate of growth per year of the cockle year-classes 1947 and 1951, both on the flat and in the creek of the Zeehondenplaat.

This difference in the rate of growth between flat- and creek-cockles becomes particularly manifest when the relative annual rate of growth is plotted against the length (fig. 14). The curve obtained from the flat-cockles' data is (a) higher, and (b) more towards the right than the creek-cockles' curve. A comparison between equally large animals from the flat and from the creek, therefore, shows a higher rate of growth, at all times, on the part of the flat-cockles.

All the same, the curves of the flat- and creek-cockles do show considerable similarity, which becomes most clearly evident when a logarithmic scale is applied to the two axes of fig. 14. It is remarkable that the lines of the flat- and the creek-cockles of one and the same year-

class run parallel, but that those of two different year-classes do not run parallel (fig. 14a). The relative rate of growth of the year-class 1947 is found to be inversely proportional to about the second power of the length; but the relative rate of growth of the year-class 1951 was greater, being inversely proportional to the 1.5 power of the length. (The lines based on the growth of cockles in other parts of the Wadden Sea show no marked parallelism; so the results of the Zeehondenplaat are probably only of local significance in this respect.)

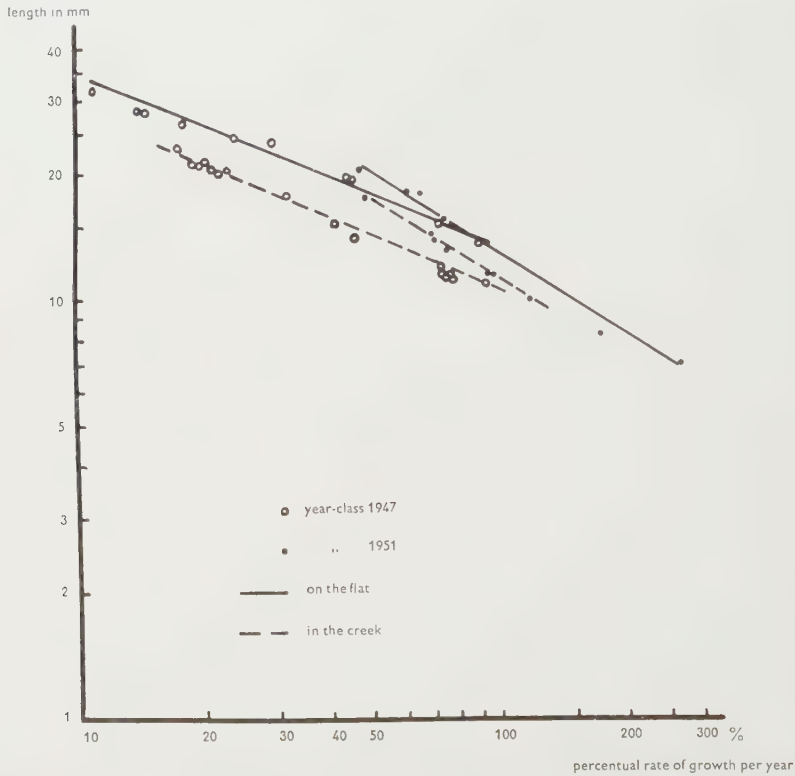

Fig. 14a. The same data as in fig. 14, but both axes of the figure with logarithmic scale.

It cann ot be readily seen from fig. 14 which animals from the various sampling places show an equally great increase in length (in mm), because the points of equally great increase in length lie on a hyperbola But when, instead of the relative rate of growth, the reciprocal value of the relative rate of growth is plotted, the hyperbolae of equal increases in length change into straight lines that pass through zero (see fig. 15).

All points, therefore, which lie on the left of such a straight line represent a faster increase in length than the points *on* the straight line.

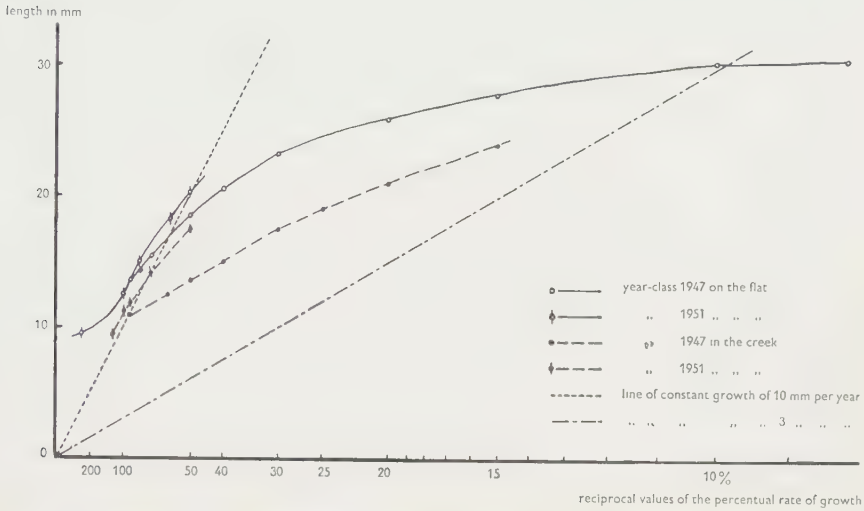

Fig. 15. Growth of the cockle year-classes 1947 and 1951 on the flat and in the creek of the Zeehondenplaat.

The reciprocal values of the percentual growth rate per year are plotted against the length of the cockles. The straight lines through zero give the relation between the length and the rate of growth, at a (hypothetical) constant increase in length of 10 mm and of 3 mm per year.

The course of the lines that indicate the ratio cockle-length: rate of growth, in one and the same sampling place, shows that the tiny cockles thanks to their enormous growth, are to a large extent able, at first, to overtake the larger animals; but according as these small cockles get bigger, the differences in relative growth are reduced to a point where they still do not quite overtake the larger animals.

This fact is also manifested in fig. 13 (p. 407); the lines rendering the annual growth of large, medium-sized and small cockles within one and the same group, show a diminishing divergence towards the right. The same principle is also expressed in the standard deviation, which, too, diminishes according as the increase in length diminishes (see fig. 16, p. 410). There is clearly a connexion between the standard deviation in length and the change in the annual increase in length.

On the Zeehondenplaat, this phenomenon—gradually decreasing spread of the length—applies not only to the 1947 year-class, but equally to the 1951 year-class, both as regards the cockles on the flat, and those in the creek (see fig. 17). Moreover, on other flats too, the principle that the smaller cockles, in the course of the years, gain on the

larger ones of the same year-class, is evidently valid. This is most clearly evident in the case of the spat which settles later in the year, and therefore enters upon its first winter while still extremely small. We have a documented example of this in regard to the Zeehondenplaat. This refers to the 1951 year-class on the higher part of the flat. A small group of 12 animals showed an exceptionally small first winter-ring (evidently this spat had not settled until August 1951). Its growth in 1952 amounted to 271 per cent., so that finally they were only a little behind the other cockles from the same sample as regards length (see table xx and fig. 14 and 17).

The results discussed so far as relating to growth are solely founded on determinations of length. We know, however, that the shape and the weight of the cockle's shell depend upon different environmental factors. Where there is no surf, and where the water is brackish and the bottom muddy, the shells are very asymmetrical, and also light in weight. On the other hand, the cockles from a surfy area, with a sandy

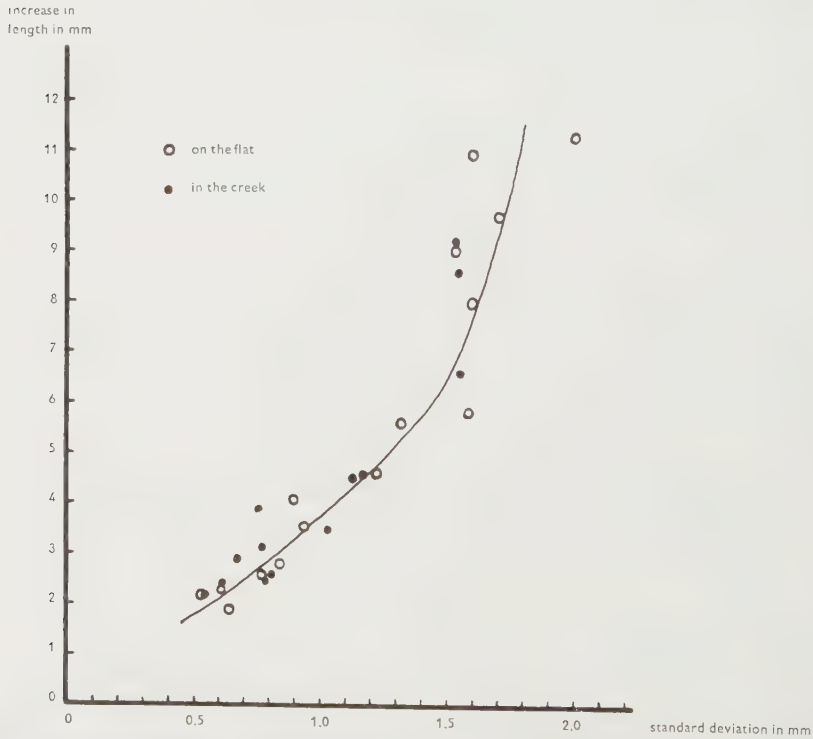

Fig. 16. Relation between increase in length and the standard deviation in the length of the cockles on the Zeehondenplaat. Each point refers to between 17 and 123 specimens (average 63 specimens).

bottom and where the water is not brackish, have a more symmetrical and also heavier shell (cf. PURCHON 1939). STEPHEN (1932, p. 64) further demonstrated that, on a tidal sandflat in Scotland, the cockles' shells were heavier according as the time of submersion was longer.

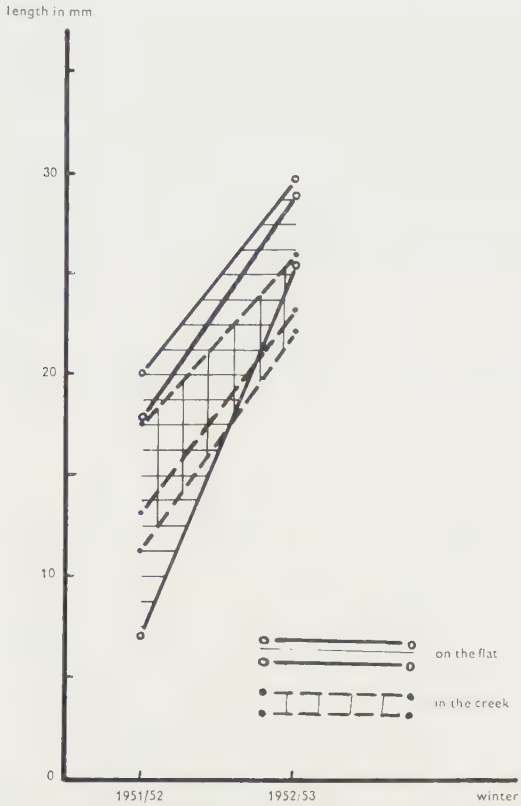

Fig. 17. Increase in length from the first to the second winter, of large, medium-size, and small cockles on the flat and in the creek of the Zeehondenplaat.

We will now, therefore, proceed to discuss in how far an increase in height, thickness, volume and weight runs parallel with an increase in length.

The increase in the *height of the shell* is found to run practically parallel with the increase in length, as we found from about a thousand measurements of the ratio length : height : thickness. As may be read from the diagram in fig. 18, the height increases in direct proportion to the length in cockles of from 2 to 20 mm length; the ratio length : height is invariably 1.14, with an average standard deviation of only

4 per cent. In cockles larger than 20 mm, however, the height increases slightly more slowly than the length, so that, at a length of 38 mm, the ratio length : height = 1.19 (with an average standard deviation of 3 per cent.).

This difference is the result of the change of shape which the shell undergoes during growth. For, whereas both valves of the shell are, in the beginning, more or less symmetrical, the shells of the larger cockles assume a slightly pointed shape on the posterior side, which affects the length more than the height (see fig. 19).

The *thickness*, i.e. the cross-diameter of the cockle, too, runs largely parallel with the length (see fig. 18); the ratio length : thickness is 1.56; with an average standard deviation of 5 per cent. With regard to the thickness, we found (at any rate in 1955) the curious phenomenon that the cockles of 6–15 mm length were, on the Zuidwal, thicker by an average of 9 per cent. than equally long animals from the Zeehondenplaat. But the animals of between 30 and 38 mm, on the contrary, were 3 per cent. thicker on the Zeehondenplaat than on the Zuidwal. We are at a loss to explain this phenomenon. It is furthermore quite remarkable

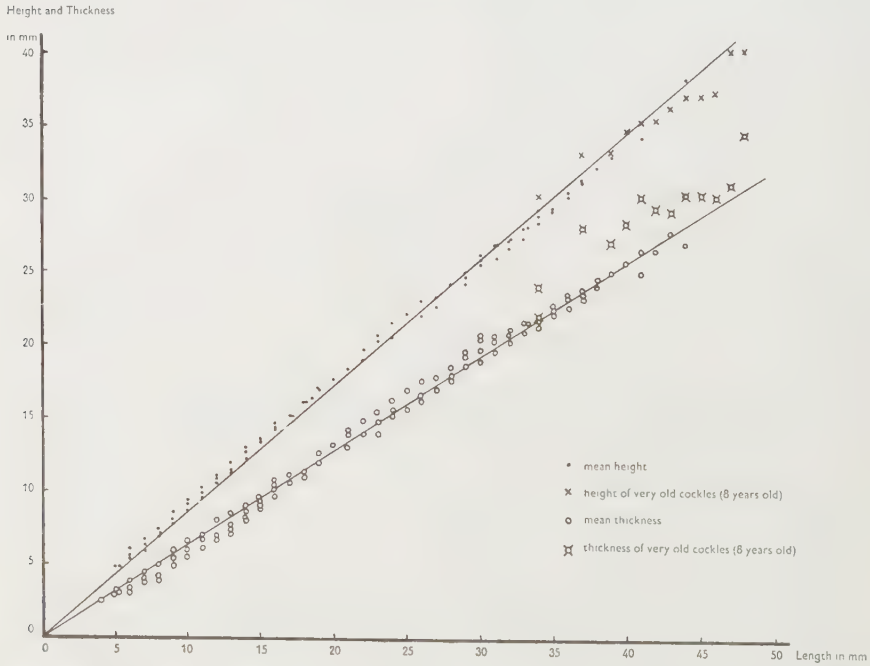

Fig. 18. The relation between length and height, and between length and thickness of over 1000 cockle-shells from the Zuidwal and the Zeehondenplaat (July-September 1955)

that about fifty very large Zeehondenplaat-cockles were exceptionally thick (12 per cent. thicker than normal). These were 8-year-old cockles, which had evidently grown more in thickness than in length during the last year or so.

We have no data concerning the ratio of the *volume* of the cockle to the length of the shell.

The *weight* is most important as an indicator of the growth of bivalves. Of the oyster, for instance, it is known (HAVINGA 1928) that the animals' growth in length is limited to a very short period, while the weight of the shell and of the meat increases fairly gradually. For the oyster, therefore, the weight is a better measure of its growth than the length.

In order to get an insight into the relation length: weight of the cockle, the dry weight of the shell and of the flesh of some dozens of samples was determined during the years 1952-1956. To this end, the cockles of each sample were divided into millimetre-categories and the shells and flesh of each category were weighed.

When the length is graphically plotted against the weight, it is natural that no rectilinear relation is to be expected. If, instead of the

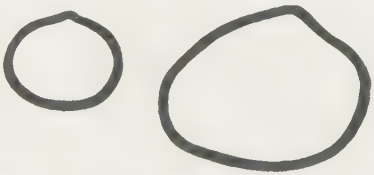

Fig. 19. The shape of the shell of a juvenile and of an adult cockle (natural size)

weight, we take the cube root of the weight, the relation does turn out more or less rectilinear; but the connexion is less close than would be expected. This result shows that it would be wrong just to take the length as a measure of growth. Further inquiry into the relation length : weight showed us that, in the area investigated by us, there were three causes which rendered this relation inconstant. Firstly, it was found not to be constant in the course of the year; secondly, the relation was a different one in cockle *spat* from that in older cockles, and thirdly, we found that the relation may also differ as between different habitats.

With regard to seasonal differences and differences in age, we have only a single reliable series. At a permanent sampling place on the Zuidwal (close to the Vangdam) cockles were collected two years in succession, and measured and weighed. The resulting data show that the increases in length, in the weight of the shell and in the dry weight of the flesh, respectively, are unequal (see fig. 20). This becomes still more clearly manifest when the cube root of the weight of the shell is

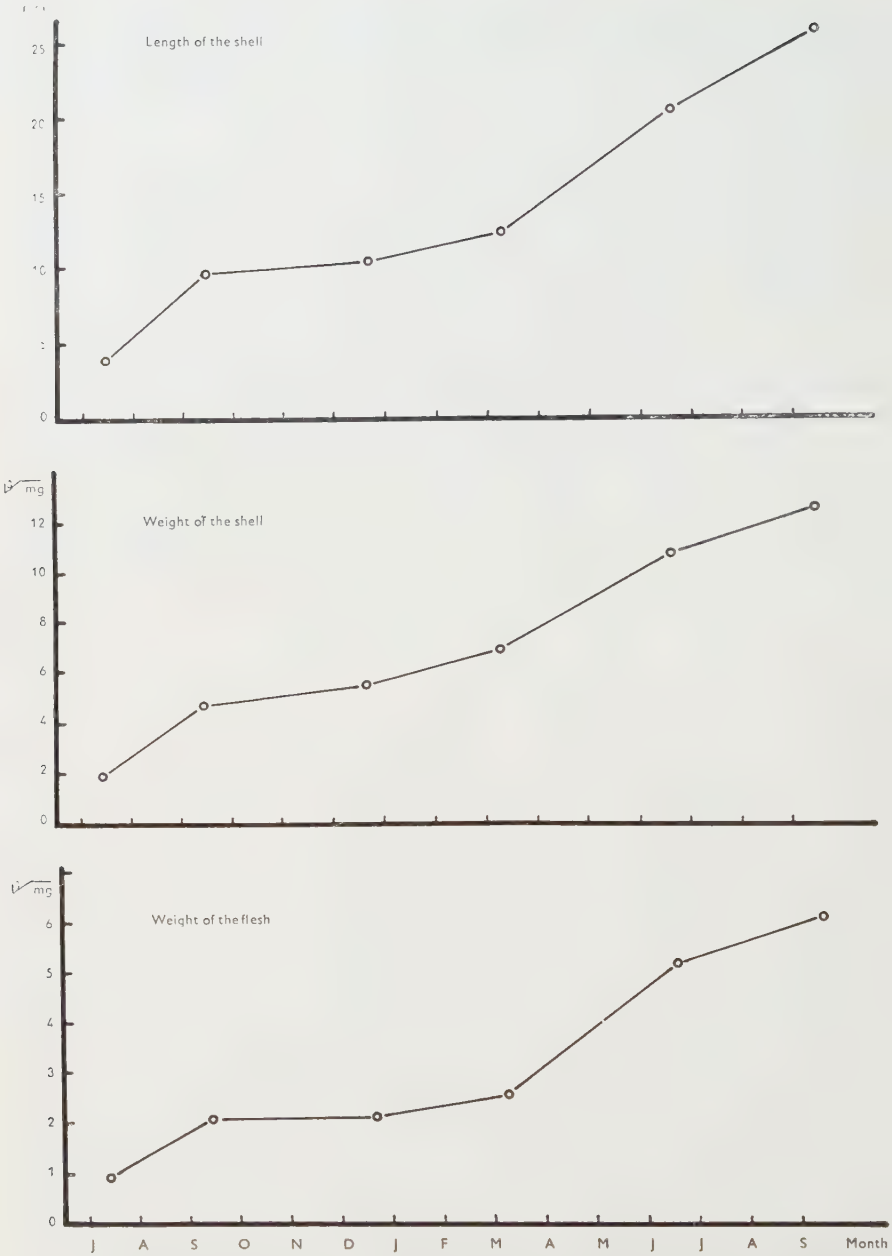

Fig. 20. Increase in length, and the third power root of weight of shell, and weight of flesh of the cockles on the Zuidwal in their first and second years (July 1954 – October 1955).

plotted against the length (fig. 21a), when it will be seen that the weight of the shell is relatively low in September of the first year, but that it steadily increases in the course of the winter until March. In early summer, during the rapid springtime growth, the weight of the shell—relatively speaking—falls slightly, especially in the larger speci-

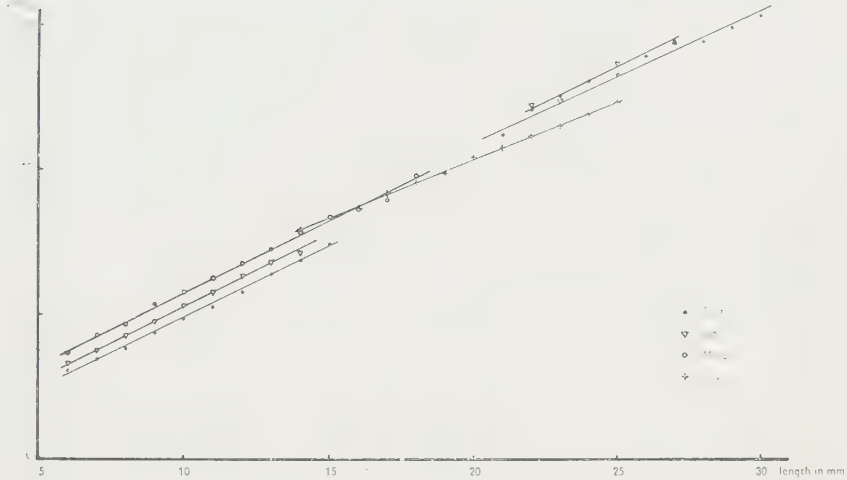

Fig. 21a. The relation length of shell : third power root of weight of shell in the first two years of the cockle's life (Zuidwal 1955–1956).

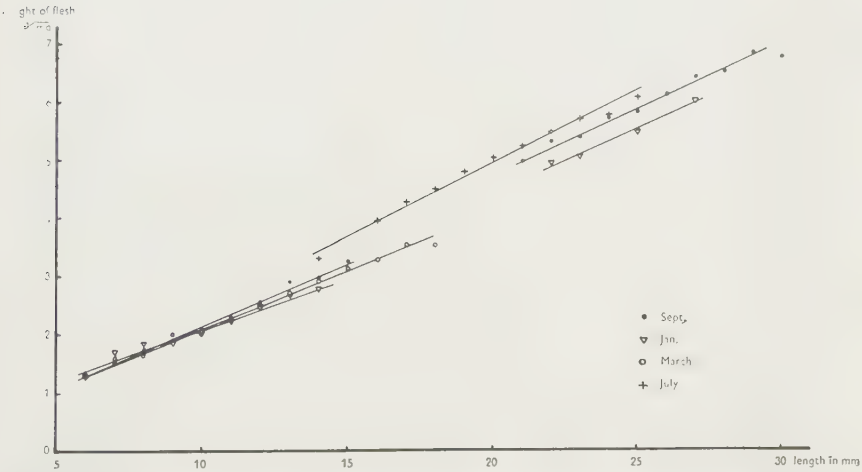

Fig. 21b. The relation length of shell : third power root of weight of flesh in the first two years of the cockle's life (Zuidwal 1955–1596).

mens; but it subsequently rises again in late summer. During the second autumn the weight of the shell hardly changes at all.

With regard to the weight of the flesh, the changes take a different course (see fig. 21b). From September to March of the first year of life there are not many changes in the weight with respect to the length. But in the early summer the weight of the flesh increases considerably, after which it decreases again—relatively—until the following winter.

The most striking thing is surely the increase in weight of the shell in the autumn and winter of the first year of life, when the increase in length largely stagnates. What also strikes one is the relative increase in weight of the flesh in springtime; i.e. in the period when the increase in length is very rapid, while, in addition, the gonads mature during this period. Even in June, the weight of the flesh is found to be still relatively high, despite the fact that the discharge of sexual products has meanwhile taken place.

With regard to the ratio between length and weight in different habitats, STEPHEN (1932, p. 64) found that, on the sandflats of Loch Fyne, the cockles have a relatively heavier shell the closer they live to the low-water level. We found a similar situation on the very high flats of the Lauwerszee. The cockles of the low parts of the flat had shells weighing about 9 per cent. more than those of cockles of the same size that lived on parts emerging about 9 hours per tide. On the low part, on the contrary, the weight of the flesh of animals of equal size was 9 per cent. lower than on the high part.

On the Zeehondenplaat we found that, on the lowest part—i.e. in the creek—the cockles were, on the average, lighter in weight than those of corresponding length on the higher part of the flat. The difference in weight between the shells was only 1 per cent.; but the weight of the flesh of the creek cockles was, in April, as much as 25 per cent. lower than that of the cockles of the same length higher on the flat. In June and November the greatest differences in the weight of the flesh between the cockles from the flat and those from the creek, were 9 per cent. or even less.

Within some months there may arise considerable differences in weight at fairly short distances, as was found during an examination on the eastern part of the Zuidwal (near the Amsteldiep), where we took two samples at a distance of 200 m from each other. One sample was collected at low-water level, where mussel spat had settled in the course of the summer, with the result that, in the second half of September 1955, we there found about 1500 small mussels of $\pm$ 14 mm per m^2. The second sample was taken 200 m higher up on the flat, where the submersion time was 9–10 hours per tide, and where no mussel spat had settled. The cockles from the low-lying sample were, on the average, slightly smaller than those from the sample taken higher up (low-lying

cockles, 31.6 mm; higher ones, 33.0 mm), while the length of the last winter-ring showed that the cockles had grown practically equally fast in length during the preceding year (both groups by 16 per cent.). We found, however, that in the lower sampling place, where the cockles had been living among the mussels, the weight of their shells was ± 8 per cent. less than that of equally large cockles in the higher sampling place, while the weight of the former's flesh was even 16 per cent. less (see fig. 22).

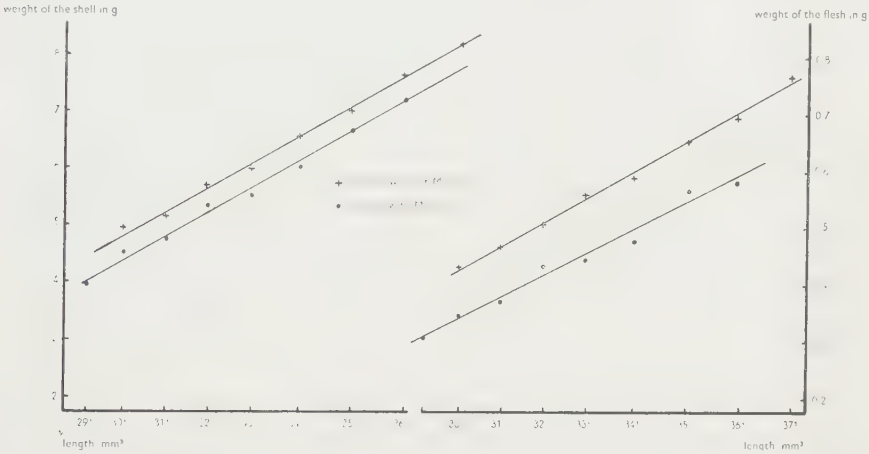

Fig. 22. The weight of the shell and the weight of the flesh with respect to the third power of the length of the shell, in cockles living in the midst of mussels, and those living away from mussels.

We finally made a brief investigation into the possibility that there might be differences, among the cockles of various year-classes and sampling places, between the number of diverging ribs on the shell. Six samples were collected on the flat and in the creek of the Zeehondenplaat, and on the Lauwerszee flat, the year-classes 1947 and 1952 being examined separately. The results showed that there were no significant differences either between the various sampling places, or between the year-classes. The average number of ribs varied from 23.3 to 23.9, with a standard deviation between 0.8 and 1.4.

If we then ask what is the best method to study local differences in growth, we may say that the study of length alone is insufficient. We can partly overcome this difficulty by comparing samples that have been collected on the same date. But even then there is the possibility that there may be differences in weight in the absence of any differences in length, and conversely. Admittedly, we found differences in weight, with the same length, on only one of the five tidal flats we investigated with regard to this point; but that is important enough to justify greater attention being given to this relation in the future.

V. WHAT ARE THE FACTORS THAT CAUSE OR AFFECT DIFFERENCES IN GROWTH ?

We now come to the question, what factors may be responsible for the observed differences in the cockles' growth. In the first place, how are the differences in size within one and the same sample to be explained? As fig. 10 (p. 375) shows, the variation in size is already considerable in the course of the first summer. In attempting to trace the cause of this variation we shall discuss the following possibilities in order of sequence:

A. The variation in size could be a result of the cockles' different ages.

B. The variation could be a result of individual differences in the rate of growth of cockles of the same age.

A. In most years we admittedly find spat of 2 mm chiefly between the end of June and the beginning of July, but spat may also be found earlier (end of May) and later (in August, cf. BAGGERMAN 1951, p. 340). It is not surprising, therefore, that a very considerable variation in size occurs already in the first summer. But as we remarked before, the spat-fall of 1947 was of short duration, as is evident from the data we collected on the Zuidwal between June 30 and September 20 of that year. By June 30, not a single small cockle had even reached a size of 1.5 mm. On July 10 a large number of animals measuring 2 mm were found (see table XXI). On July 18, we still found one or two little ones of 2 mm, all the others were greater. We may assume, therefore, that the entire spat-fall in that year was limited to a period of less than 2 weeks. Notwithstanding the fact that the entire spat fell within such a short period, the largest animals measured 24 mm, the smallest ones 15 mm by the end of their first year. Is it possible for such a great difference in length to be based exclusively on a difference in age? If it is true that the largest animals are the oldest, and the smallest the youngest, then it may be expected that a calculation, covering the entire summer, of the daily percentage of growth of the largest and the smallest cockles, would yield a fixed ratio between these percentages and the length categories.

Now as table XXI shows, the largest animals, in developing from 3 to 8 mm, grew 12.3 per cent. per day, but the smallest ones, during the same development, by only 5.2 per cent. Above 8 mm, too, the "forerunners" are seen to have grown continuously faster than the "stragglers". This, however, implies that the variation in size cannot possibly be based on the differences in the dates of birth alone.

It might be argued that the conditions for growth may possibly become worse as the season advances. Indeed, as we have seen already, the adult cockles do not grow much after July. Then, however, one has to assume that in August the larger spat grows at a relatively high rate,

but that the smaller spat shows a typically stunted growth. This does not seem very plausible, and, therefore, we do not believe that the differences in size could be the result of differences in age.

TABLE XXI

The average, maximum and minimum size of the cockle population on the Zuidwal mud-flat in 1947, and the calculated values of the mean daily growth in different periods

1947	number of specimens	average size in mm	standard deviation in mm	percentual growth per day	percentual growth per day	max. size in mm	percentual growth per day		percentual growth per day	minim. size in mm
10 July	±100	2	(?)			3	12.3			
18 July				8.6	9.3	8	6.9			2
28 July	65	9.5	(2.1)			16		3.1	5.2	
18 August				1.3	0.8	21	1.3			10)
20 Sept.	82	19.5	(2.0)			24	0.4		(1.2)	(15?)
Winter	±100	19	(?)			24				15

B. The second possibility is that the variation in size is a result of individual differences in the animals' rate of growth. One might think, in this connexion, of congenital differences; but although these may admittedly play some part, they cannot be very important, since it was found that a cockle that has remained small in a given year usually makes up its leeway, to a large extent, the year after, while extra large cockles generally lose most of their lead the next year. This is a fact which struck us again and again during this investigation, and which is clearly illustrated in table xx (p. 406). It is due to the fact that the smaller the cockle, the greater is its rate of growth.

It might also be possible that individual differences in the rate of growth arise as a result of the frequency with which the animals are washed out, and of the duration of the transport. As already mentioned above, the youngest spat—at any rate in more or less exposed places—is repeatedly transported by the current. No doubt this transport will be of longer duration on some occasions than on others. Since we know from ORTON's (1926) investigation that every manipulation he subjected his cockles to resulted in a "disturbance-ring" being formed on the shell, it seems very likely that the growth of the spat stagnates temporarily after transportation. This would imply that the differences in length should be greater on a very much exposed flat than on a sheltered one, where there is hardly any transportation of spat. To clear up

this point, we shall compare the variation of individual linear measurements of two samples on the Zeehondenplaat, viz. one sample from the high, exposed part, and one from the more sheltered creek; in addition we may include in this comparison a sample from the very sheltered Ameland mud-flat. As a measure of the variation in size we shall take the standard deviation of the variable. Now we do not know whether the standard deviation of the length is a function of the animals' age, or of their increase in length. We shall therefore consider both possibilities. On the assumption that the standard deviation is a function of age, we must base our argument on cockles of equal age (see table XXII). We shall then find that the standard deviation is far greater on the Zeehondenplaat than on the quietly situated Amelander Slik; but on the Zeehondenplaat itself there are no significant differences between the exposed high part and the sheltered creek.

TABLE XXII

Standard deviation of the length of the first winter-ring of samples from the high part and the creek of the Zeehondenplaat, and from the Amelander Slik

	length 1st winter-ring	standard deviation $\sqrt{\frac{x^2}{k-1}}$	numbers
High part Zeehondenplaat	19.2 mm	2.0 mm	38
Creek Zeehondenplaat	13.9 mm	1.8 mm	98
Amelander Slik	14.0 mm	0.8 mm	117

If, however, the standard deviation is a function of the increase in length, we must base our argument on animals that show an equal increase in length. For this reason we have compared in fig. 23, for the three samples referred to above, the annual increase in length, per group of individuals of one particular length-category, with the standard deviation in length. As this figure shows, there is not, in fact, any distinct difference in standard deviation between the three samples. This means that—whether the standard deviation of the length is bound up with the animals' age or with their rate of growth—the transportation to which the youngest spat is subjected cannot, in any case, be of any influence on the variation in size.

Finally, there is the possibility that the variation in size is affected by purely local differences in the micro-milieu; differences which may be of a perfectly temporary nature, such as the formation of a sandy ridge, or of a more permanent nature, as the presence of a hydroid colony. We have not, however, any indication as to what environmental factor might be the most important in this respect.

Summarizing, we may say that the variation in size found within one and the same sample can at most be due partly to the age-differences

between the cockles; the variation in size cannot be explained by congenital differences in the rate of growth of the cockles; *neither* is it a result of their being transported across the flat. The obvious conclusion is that the differences in growth should be attributed to differences in the micro-milieu, which differences may be either of a temporary or a permanent nature. This implies that the differences in length found within one and the same sample are, in principle, caused by the same

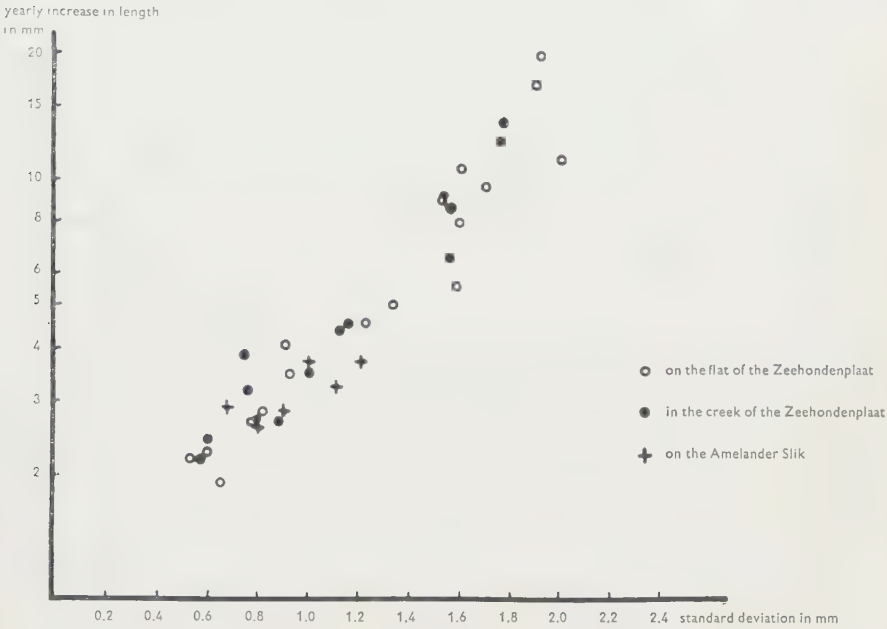

Fig. 23. Relation between yearly increase in length and the standard deviation of the variable, in the cockle year-class 1947.

factors as the differences in length between different samples. This is, for our investigation, a considerable advantage; for it eliminates the chance that, in examining the influence of environmental factors on growth, this influence might be traversed by factors of a different nature.

We shall now endeavour to ascertain what factors may be responsible for the observed differences in the rate of growth as between samples from different environments. Since our comparison will invariably involve cockles of one and the same year-class, we may, here again, regard differences in length as being due to differences in growth—at any rate in the case of such year-classes as 1947, when the entire spat-fall took place within a brief space of time (i.e. early summer). On the other

hand there have also been years when part of the local year-class consisted of "secondary spat", i.e. spat born about 2 months later than the majority. But since it was found that the secondary spat follows the same growth-curve as the majority of the spat (fig. 14, p. 407), it is clear that, after just over a year, an age-difference of a couple of months is no longer of much influence on the average length of the cockles in the sample.

1. *Influence of the duration of submersion*

Considerable stress is generally laid in the literature on the importance of the duration of submersion for the cockles' growth, larger cockles being found when the submersion-time is longer (STEPHEN 1930, THAMDRUP 1935, KREGER 1940, SMIDT 1952, and others). In our own investigation, too, we found that the cockles as a rule grow faster, the longer the duration of submersion. But we also repeatedly found exceptions, the most striking being on the Zeehondenplaat, where the largest cockles were found on the highest part of the traverse. The differences which we found in this respect in the Wadden Sea, however, become explicable when one considers both the profile of the flat and its situation with respect to the low- and high-water levels (see fig. 2, p. 355).

On the low-lying flats, which emerge at most during 3-4 hours per tide, one finds hardly any difference in growth between cockles around the low-water level and those on the top of the flat. For instance, SW of our traverse on the Zeehondenplaat, in an area which is exposed to the flood, the cockles around the low-water level are of exactly the same size as the animals on the top of the flat. We found the same thing on the Paardenhoek, and at the Kop van de Staart van Schieringhals. (On the sheltered side of these flats the cockles around the low-water level are, as a rule, even smaller than those on the top of the flat; see table XXIII.)

TABLE XXIII

Average length of the cockles on the lower and on the higher parts of flats whose high parts emerge at most during 4 hours per tide

	date	average length in mm on the low part	average length in mm on the high part	difference in length
Zeehondenplaat	June 24, 1949	32.5—25.2 *	32.2	−1 %–+28 %
Paardenhoek	July 7, 1949	27.1	31.1	+14 %
Kop van de Staart van Schieringhals	July 25, 1949	33.9	33.8	0 %

* samples from the south side of the Zeehondenplaat and from the creek, respectively.

When, on the contrary, parts of the flat are extraordinarily high, so that the time of submersion is at most 2-3 hours per tide (Lauwerszeewad, Amelander Slik, Kikkerplaat, Grienderwaard, Zuidwal, Balgzand), then the cockles on the highest parts are found to remain smaller by 20–40 per cent. than those in the lower-lying places (table XXIV).

TABLE XXIV

Average length of the cockles on the low- and on the high-lying parts of flats whose high parts emerge at least during 9 hours per tide

	date	average length in mm on the low part	average length in mm on the high part	percentual differences in length
Lauwerszeewad	March 25, 1953	20.0	16.1	—20
Amelander Slik	Sept. 27, 1949	24.0	18.5	—23
Kikkerplaat	Sept. 28, 1949	28.1	22.3	—21
Grienderwaard	Sept. 29, 1949	29.9	17.3	—42
Balgzand	Sept. 17, 1947	12.4	9.0	—28
Zuidwal	Sept. 20, 1947	15.3	13.2	—13

The importance of the duration of submersion was especially evident on the evenly sloping Balgzand flat. The length of the cockles was found to be constant over a long distance upwards from the low-water level; but beyond that the cockles became gradually smaller (table XXV).

TABLE XXV

Length of the cockles on the Balgzand flat, from the low-water level to the high-water level. 17th September 1947

	sample no.	submersion-time per tide	average length in mm
Low	1	11.5h	12.3
	2	9h	12.4
	3	8h	12.3
	4	7h	12.7
	5		12.4
	6	5h	11.8
	7		11.9
	8	4h	11.5
	9		10.9
	10	3h	9.4
	11		9.0
High	12	2.5h	9.0

That this is not equally clearly manifest on all high-lying flats is due to the fact that there are only very few flats which have such a gradually upward-sloping profile as the Balgzand flat (see p. 356). The majority

of highly situated flats, in fact, have a fairly steep profile near low-water level and there, differences in the length of the cockles as between the samples can accordingly be demonstrated. But higher up these types of flat run practically horizontal. On this horizontal part the samples hardly show any differences in the cockles' length, as is clear from the data obtained from the Amelander Slik, the Kikkerplaat, and the Grienderwaard close to the Pijp (table XXVI).

TABLE XXVI

Length in mm of the cockles at points situated between low-water level and the highest point of the flat

sample no.		Amelander Slik 27-9-1949	North part of Kikkerplaat 26-9-1949	SE part of Kikkerplaat 27-9-1949	South part of Kikkerplaat 28-9-1949	Griender-waard 29-9-1949
Low	1	24.0	23.0	24.1	28.1	25.2
	2	23.9	23.0	22.4	28.0	23.3
	3	24.0	22.9	22.0	27.0	22.9
	4	22.7	22.0	22.2	25.9	23.0
	5	20.8	21.5	21.8	25.0	22.8
	6	18.8	22.0	21.7	24.3	23.0
	7	18.9	21.8	22.1	23.0	
	8	18.5	21.9	21.0	23.1	
	9	18.5	21.9	21.0	22.5	
	10	18.5	21.8	21.7	22.4	
	11	18.6	21.8	21.3	22.4	
High	12	18.5	21.9	21.4	22.1	

Hardly anywhere in the Wadden Sea did we find a flat where the length of the cockles decreases at an even rate from low- to high-water level. Such a gradual change in connexion with the duration of submersion is only to be expected on a flat having a steep and high profile, after which it rises at an even slope, as is the case on the Lauwerszee-wad (see fig. 2). There, samples were taken at 4 points; the lowest sample lay at low-water level; the following three (at 100 metres' distance from each other) all lay dry for more than 6 hours per tide. And, in fact, the length of the cockles decreased at an even rate from low- to high-water level (table XXVII). On the south part of the Kikkerplaat, too, the length decreases gradually as the cockles are found further away from low-water level (see table XXVI). Maybe the profile of the flat at this point corresponds more closely to the Lauwerszee-wad type than to that of the Amelander Slik.

As the Danish flat of Skallingen, which THAMDRUP (1935) investigated, has a profile strongly resembling that of the Lauwerszee-wad (see p. 356) we are not surprised that THAMDRUP concluded that the length of the cockles invariably increases evenly with the duration of submersion.

The high location also of other Danish flats was probably the reason why SMIDT (1951, p. 81) stated that the cockles on the flat invariably grow less fast than those in the channels. But when SMIDT goes on to say that the cockles grow faster the lower they lie on the flat, then this is not in accordance with our experience. The figures which he gives do not constitute conclusive evidence in this respect, since his data show that he has only compared the cockles from the *higher* part of the flat with the cockles from the channel. That these cockles from the channel grow faster than those at the top of the flat is perfectly understandable; it does not, however, prove that these cockles also grew faster than the animals on the lower part of the flat, which were left out of consideration in his comparison.

TABLE XXVII

Length in mm of the cockles of the Lauwerszee-wad, at 4 points located between low- and high-water level. (25th March 1953)

sample no.	1	2	3	4
Submersion-time per tide	±12hrs	±4hrs	±3hrs	2.5hrs
Average length in mm	20.0	18.7	17.5	16.5

Although, therefore, a correlation was found, on the high-lying flats all over the Wadden Sea, between the length of the cockles and the duration of submersion, we still do not assume that either submersion or emergence, as such, can have any direct influence on the growth of the cockles. But the consequence of long emergence is that different abiotic factors may reach extreme values during this period, values that will never be reached during submersion. This applies to salinity, temperature, light, and oxygen. And furthermore, what is far more important still, emergence of the flat hinders the cockle in taking in food.

2. *Influence of salinity*

It is a striking fact that the cockle reaches a greater length the more strongly the animal is under the influence of Atlantic water. According to WINCKWORTH (1921), cockles from Orkney reach an average length of 60 mm, and on the west coast of Scotland STEPHEN (1930) found cockles of over 50 mm. JOHNSTONE (1899, p. 2) says in this connexion: "... the largest specimens are found only in areas far removed from the influence of fresh water".

On the other hand, it is a wellknown phenomenon that cockles in brackish water remain small in size (e.g. REMANE 1934, p. 66). This has led to the conclusion that low salinity inhibits growth (e.g. LARSEN

1936, p. 14). Although this sounds very plausible, there is no decisive proof—as far as the present writer is aware—that salinity as such really influences growth. But this problem is hardly of importance in respect of the areas in the Wadden Sea which we investigated. In the Wadden Sea, at very short distances—such as between the samples from the Zeehondenplaat—only differences in salinity of, at most, a few hundredths or tenths of ‰ can be found; and even these very small differences are not constant. Neither are there any constant differences in salinity between the different flats investigated by us, although, outside the estuary or south of Ameland the salinity is a few ‰ higher than in the areas nearer the sluices of the Afsluitdijk (Enclosing dyke of the former Zuyderzee), as is shown by the salinity investigations made in the course of a number of years (Dr. L. F. Kamps, verbal communication).

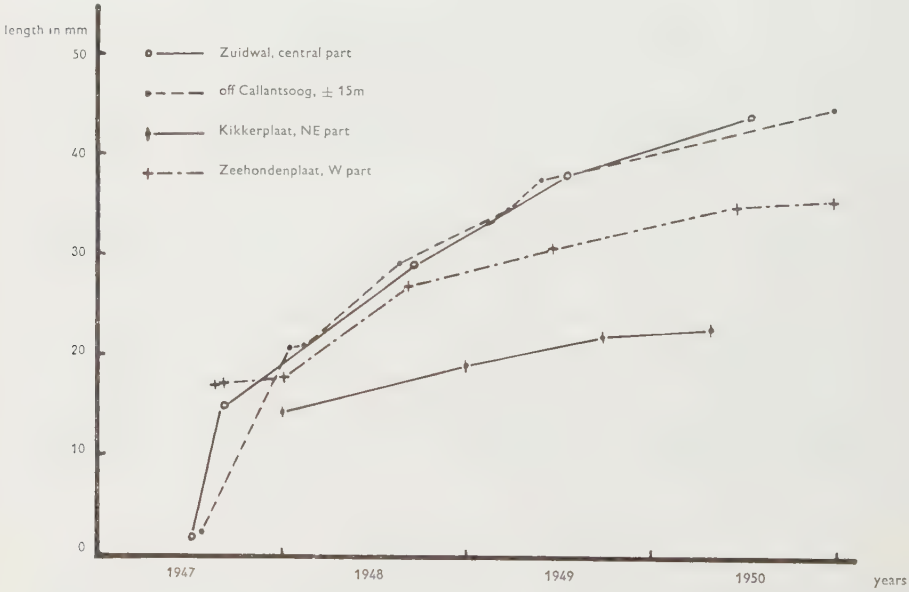

Fig. 24. Growth of the cockle year-class 1947, in and outside the Wadden Sea.

There is no doubt that the cockles in the North Sea live in water with relatively the highest salinity; and, in fact, these cockles do grow very fast (fig. 24). At the Kikkerplaat, near Ameland, however, the salinity of the water is not much lower; and there the cockles grow more poorly than any other cockles that we have come across. The salinity of the water near the Zuidwal and the Zeehondenplaat is about 2‰ lower than in the North Sea; and in the former the growth of the cockles is about the same, in the latter it is half-way between that of the North Sea

and of Ameland. It is improbable, therefore, that differences in salinity exercise any important influence on the animals' rate of growth in the area investigated.

None the less, it is possible that extreme differences in salinity, caused either by excessive evaporation or unusually copious rainfall on an emerged flat, might exercise an adverse influence on the growth of the cockles. In fact, spat smaller than 2 mm will very likely find its growth inhibited (cf. p. 383). Our own data, however, are insufficient to justify either the denial or the affirmation of such a short-term influence of abnormal salinities on the size of the cockles in any part of the area investigated by us. If such an influence existed, it would be of a strictly local and, therefore, subordinate significance.

3. *Influence of temperature*

Since the rate of growth of organisms rises, within certain limits, with the tempearature, attention must be given also to this factor when inquiring into the causes of differences in growth.

We shall now inquire, therefore, whether the differences in the average temperature of the water may have an effect on the growth rate in various locations, for the cockle year-class 1947.

The temperature of the water in the shallow Wadden Sea will, generally speaking, differ from that of the North Sea. From about the middle of March to the beginning of September the average temperature of the water of the Western Wadden Sea is 1°C higher than that in the North Sea, and up to 3°C lower in the six winter months (Postma and Verwey 1950, p. 13, fig. 3).

We find that the cockles in the Wadden Sea have generally grown faster according to whether the water in a given area is more subject to the influence of the North Sea. The biggest cockles were found by us outside the Wadden Sea proper. When one wishes to ascertain the influence of the water temperature on the animals' growth in the North Sea and the Wadden Sea, one should not, of course, compare cockles from the North Sea with those from emerging flats, since the emergence in itself entails various consequences with respect to growth. For this reason we have compared cockles from the North Sea off Callantsoog, 15 metres deep, with cockles from the low-water level of a number of flats (see table XXVIII). As will, in fact, be seen, the cockles grow fastest in the North Sea. But we cannot conclude from this that the more extreme values of the temperature in the Wadden Sea area are unfavourable to the animals' growth; for at the NE edge of the Zuidwal, where the mean temperatures in summer and in winter are without a doubt far more extreme than in the deeper water off Callants-

oog, the rate of growth is hardly lower than in the North Sea.

In view of the fact that any difference in average temperature between two points located at a short distance from each other will naturally be much smaller still, it appears improbable that differences in growth on, for instance, different parts of one and the same flat should be caused by the temperature. In fact, there is nothing to support such an assumption.

TABLE XXVIII

Length of the first and second winter-ring of cockles from different areas

cockle year-class 1947	average length of first ring in mm (number of specimens)	average length of second ring in mm (number of specimens)
Callantsoog 15 m deep	21.0 (46)	31.3 (43)
NE edge of Zuidwal	20.9 (15)	31.1 (15)
N edge of Zuidwal	18.2 (54)	30.0 (60)
SW of Paardenhoek	19.7 (90)	29.0 (90)
S edge of Zeehondenplaat	19.4 (42)	28.4 (51)
SW edge of Kikkerplaat	17.5 (44)	28.6 (44)

We shall now endeavour to trace the influence of extreme temperatures on the growth of cockles. On the top of the emerging flats the cockles are exposed at low water to the action of the air temperature, which, of course, in winter reaches much lower, and in summer much higher values than that of the water.

As is evident from our investigation discussed on p. 385, the Wadden Sea cockles tolerate low temperatures down to —1.7°C. It is probable that in the period 1947–1952 the cockles were nowhere exposed to such a low temperature. During one of the coldest periods of these years, i.e. on February 28, 1948, we made some observations on the Zuidwal flat, which at low water was covered partly with a crust of ice and partly by freezing sea-water, while at a few points the bottom lay completely dry. Nevertheless we failed to demonstrate any bottom temperature lower than —0.8°C.

Although the experiments admittedly showed that temperatures down to —1.7°C. are tolerated without any visible harmful effect, such temperatures might nevertheless have some temporary or lasting after-effect. That temperatures around freezing point are, indeed, bound to have a considerable influence on the growth of cockles, is clear from the fact that, in the relatively severe winter of 1953/'54, a much more distinct winter-ring was formed than in the mild winters from 1947 to 1952 inclusive.

As regards high temperatures, our experiments (described on p. 389)

showed that a few minutes' exposure to a temperature of around 35 °C is lethal to the cockle. Exposure of some hours to 33 °C causes the death of part of the cockles—not at once, but after some days or even weeks. Temperatures below 31 °C—even when the animals had been exposed to them for hours—never proved fatal, which, however, does not mean that unduly high temperatures could not have a temporary or lasting inhibiting influence on growth. Now in the Wadden Sea temperatures exceeding about 25 °C never occur; neither did we ever find any lethal thermal values on parts of the Wadden bottom that had emerged. But what does occasionally happen in summer is that the temperature in very shallow, isolated puddles that are sometimes formed on the flats during emergence runs up to a very high value; on one occasion, in fact, we even registered a water temperature of 32 °C, although, at 1 cm depth in the bottom, it was no higher than 29 °C.

On the high-lying part of the Zuidwal we made some observations in which samples of cockles from a small, very shallow puddle were compared with those from a small mound situated about half a metre away. The position of such puddles and mounds is stable, at any rate during the summer, as the unbroken diatomic layer covering the bottom of the higher parts of the flat remains undisturbed for months on end.

The animals in the puddle were generally found to be just a little larger than those on the mound (see table XXIX), so that, even in a very hot summer such as the 1947 one, no harmful effect of any extremely high temperature on the cockle spat in the shallow puddles could be ascertained. One wonders what may be the cause of the differences in growth. We suspect that, at a given moment during ebb-tide, the mounds emerge, while the water that is still draining off takes its way along the lower parts, where the cockles will then have more time to collect their food.

TABLE XXIX

Comparison of cockle samples from adjacent places; one being on a mound, and the other in a puddle. Zuidwal, September 16, 1947

	on the mound		in the puddle	
pair of samples	average size in mm	numbers of specimens	average size in mm	numbers of specimens
No. 1	12.4	336	13.8	401
No. 2	13.7	177	15.0	102
No. 3	13.9	154	14.1	163
No. 4	14.4	94	14.7	120

We may therefore conclude that differences in the animals' growth as between different locations cannot have arisen owing to differences in temperature.

4. *Influence of light*

ORTON (1926) has published data from which it is clear that cockles living free on an open flat do not grow any faster than those placed on the same flat, but kept inside an almost dark wooden box.

Neither have the experiments mentioned on p. 394, relating to the influence of light on the growth of the cockle, produced any evidence of the existence of such an influence.

It therefore appears out of the question that differences in the rate of growth, found in the areas investigated, could be attributed to light influence.

5. *Influence of the available quantity of oxygen*

So long as a flat is submerged any danger of O_2 deficiency is precluded. Even when a layer of detritus has temporarily spread over the cockles this layer is so light that it appears extremely improbable that deficiency of oxygen could arise underneath this layer.

VAN DAM (1938), dealing with *Mya*, put forward the plausible argument that the quantity of water which *Mya* pumps through its gills is primarily required to assure the animal of a sufficient quantity of food, while its need of O_2 could be met by a much smaller quantity of water. We do not think that conditions are essentially different in the case of the cockle, and that cockles are troubled by oxygen deficiency during submersion.

Things are entirely different, however, when the flat emerges. The cockles then close up completely or nearly so, when a tiny edge of the mantle remains visible. So long as there is no water on the flat the mantle cavity of the cockle is ventilated by the air (LINKE 1939, p. 275); but according to THAMDRUP (1935, p. 63) the respiratory intensity is lowered.

On the Zeehondenplaat there is no noticeable influence whatsoever of any oxygen deficiency, since the cockles there grow faster on the top of the flat than in the creek (see fig. 12, p. 404). But this flat emerges only for a few hours per tide.

Looking at the higher situated flats we find a different state of things, — for instance high up on the Zuidwal flat. Comparing the cockles from emerging and non-emerging places in each other's immediate vicinity we occasionally find striking differences in size. An example of this are the four groups of cockles mentioned above (p. 429) from two points each time, about half a metre apart. Their differences in length might perhaps be due to deficiency of oxygen to which the cockles on the mound would be subjected during the longer period of emergence. As, however, the amount of water, pumped through the gills of a bivalve, is

probably not primarily required for the animal's oxygen, but for its food supply, it seems more likely that during a prolonged emergence the cockle suffers most from lack of food—and not from oxygen deficiency.

It is clear that further data will be needed to demonstrate that weakened respiratory intensity of cockles during the emergence of a flat might be of influence on their rate of growth.

6. *Influence of the structure of the substratum*

Speaking very generally, we may say that cockles grow faster on a pronouncedly sandy bottom than on a muddy one. On the Zeehondenplaat too—as may be read from fig. 4 (p. 362)—there is a certain correlation between the structure of the bottom and the size of the cockles; it is in the creek (points 3–6), where the cockles are smallest, that the finest sand and most mud is found in the bottom, in addition to which a remarkably large quantity of detritus is deposited in the creek.

From an experiment made by ORTON (1926), however, one may deduce that the structure of the bottom does not affect the rate of growth of cockles. ORTON had placed on the flat a box containing a number of cockles. At first there was a small layer of sand in that box, but it was not long before this was covered by a thick layer of fairly soft mud. And the growth of the cockles in this mud was perfectly normal as compared to the growth of the cockles on the flat around the box.

In order to test the possible influence of the substratum on the growth of cockles in the Western Wadden Sea, we have collected in table xxx (p. 432) as many data as possible from the various flats. These data refer to sampling places whose maximum emergence time is 6 hours per tide, so that the submersion time cannot have been of any influence. The cockles we measured all belonged to the year-class 1947.

When comparing the size of the cockles from the various flats we do find, in fact, that there cannot have existed any close correlation between growth and bottom structure. If we may expect, on the basis of the Zeehondenplaat situation, that cockles grow fastest where the sand is coarsest, then Griend and the Zuidwal ought to have the largest cockles. And indeed, the Zuidwal cockles are among the fastest "growers"—the Griend ones, on the contrary, among the slow ones.

There might be a tendency, on each separate flat, for the cockles on the part with the coarsest sand to grow fastest;—yet again, that does not apply either to the Zuidwal or to the Staart van Schieringhals.

Neither is the relation any clearer between the mud content in the bottom and the rate of growth. True, there is a tendency on the flats separately, for the cockles to be smaller the muddier the bottom is; but this is contradicted both by the prosperous growth also on the most

muddy part of the Zuidwal and by the poor growth on the Griend flat, which has a small mud component.

TABLE XXX

The length of the cockles and the structure of the bottom on different flats in the autumn of 1948

date	flat	samples no.	average length of cockles in mm	median grain size in μ	mud in gr/l
23-9-1948	Zeehondenplaat	7–16	26.3	113	4
		4–6	20.7	87	126
22-9-1948	W part of Zuidwal	2–7	29.6	99	70
		9–17	29.6	132	7
16-11-1948	E part of Zuidwal	2,3,9–13	28.8	134	10
		4–8	24.3	129	47
8-9-1948	Paardenhoek	1–9, 12–26	28.1	128	12
		10,11, 27–32	25.0	113	27
5-9-1948	Staart van Schieringhals	1–10, 17–19	26.9	93	14
		11–16	24.3	95	22
7-9-1948	Kop van Staart van Schieringhals	1–12	28.8	103	11
4-9-1948	E of Griend	2–8	23.7	132	25
		32–40	20.6	90	13
4-9-1948	S of Griend	1–4	24.4	161	13

We admittedly found a low rate of growth on the muddy parts of the Frisian flats; but these flats are situated so high up that their short submersion time alone is sufficient explanation of the cockles' poor growth rate.

7. *Influence of floating detritus, deposited temporarily on the bottom*

While there does not appear to exist a fixed correlation between the substratum and the growth of the cockles, we strongly had the impression that there does exist a relation between the rate of growth and the quantity of floating detritus which, dependent upon the direction and force of the current, is temporarily deposited on the bottom. The clearest picture of this depositing of detritus we got on the Zeehondenplaat. At low water, a layer of peat flakes, plant remains etc., a few cm thick, lies on the muddy slope of the fairway the Oude Vlie, below low-water level and down to a few metres' depth. As the water rises, part of this detritus is carried by the flood-water on to the flat, remaining there

at first in the shallow creek on that flat—just where cockles grow badly. As the flood-current becomes stronger the detritus wells up again from the bottom of the creek, and is brought higher up onto the flat. At high water the detritus sinks to the bottom, spread out over the whole flat. Later on it is carried back again by the receding tide. When the water has subsided, most of the detritus from the creek has already disappeared, and lies on the low-water level of the Oude Vlie. These observations were made from a dinghy above the Zeehondenplaat and were confirmed by the results yielded by a number of dug-in glass jars on the flat (see fig. 4, p. 362).

On the low part of the Zuidwal we also dug a series of glass jars into the bottom. Here again, a correlation was found to exist between the growth of the cockles and the quantity of detritus deposited in the glass jars (table XXXI). At the points where the largest quantities of detritus had been deposited (samples 1, 2, 9, 15, 16), the growth-rate was poor, at least, as far as there were any cockles present at these points. At the points with little detritus, on the contrary (samples 12, 13, 14), the growth-rate was high.

TABLE XXXI

Length of the cockles and the height of the column of detritus deposited into dug-in glass jars during a single tide. Zuidwal, September 22, 1948

	sample no.	average length of cockles in mm	quantity of detritus in cm
Low	1	—	11
	2	25.9	10
	3	28.3	5
	4	27.9	5
	5	27.9	5
	6	27.0	4
	7	27.5	5
Highest point	8	27.3	6
	9	26.1	9
	10	27.0	6
	11	28.3	4
	12	29.1	1
	13	29.0	2
	14	29.2	1
	15	26.0	11
Low	16	—	12

A few series of observations made from a dinghy, on the east part of the Zuidwal, on the Balgzand and on the Paardenhoek, strongly suggest that there, too, a correlation exists between the growth of the cockles and the quantity of detritus deposit lying there during part of the tidal

period. These observations, however, were not supplemented with any quantitative determinations.

We should here remark that the quantities of detritus on and in the bottom do not invariably run parallel. In the sheltered places there are substrata containing a large quantity of detritus (Balgzand), without a layer of floating detritus being washed over it at each tide. Conversely, there are in exposed places substrata without any detritus (low part of Zuidwal), across which there nevertheless passes, for a short time, a fairly thick layer of detritus—as was shown by the above experiment with the dug-in glass jars.

One wonders in what way to conceive the growth-inhibiting action of a temporary layer of detritus. The idea of a "smothering blanket" is probably quite wrong, since the material lies extraordinarily loose on the bottom and is, moreover, in constant movement. We imagine, however, that the cockles, in inhaling water to satisfy their need for oxygen and food, are hampered by the loose detritus flakes, in the same way in which material suspended in the water may inhibit the intake of food (see below).

8. *Influence of material suspended in the water*

Dr. L. F. Kamps, at Baflo, mentioned to us that during his investigation into the role played by shell-fish in the sedimentation of mud along the Groningen coast he had a strong impression that cockles remain smaller as the water is richer in silt. This communication induced us to make an investigation on the mud-flats near the watershed along the coast of Groningen and Friesland, where we ascertained that the cockles there, in fact, were remarkably small. This, however, was partly due to the low average age of the cockles in those parts: we nowhere found any cockles older than two years. In addition, the flats there were so high that the short submersion-time, too, must have acted as a brake ongrowth. There was only a single flat found by us in this area that emerged for a shorter period (a few hours per tide); it was a sandy flat near the fairway of the Zwarte Haan. There, the cockles had their first winter-ring at 13.6 mm and their second winter-ring at 22.2 mm, which means a rate of growth more or less corresponding to that of the cockles in the creek of the Zeehondenplaat. The weight of the shell, relatively to its length, was also similar; but the relative weight of the flesh was, near the Zwarte Haan, still 20–40 per cent. lower than the—already low—relative weight of the flesh of the cockles from the creek of the Zeehondenplaat.

The growth-rate in the watershed area along the coast, therefore, was poor indeed.

It is wellknown that the total quantity of material suspended in the water increases as one penetrates further from the tidal inlet into the Wadden Sea (KAMPS 1950, p. 112); but this increase need not be coupled with any increase in the quantity of food suitable for the cockle. The data collected by POSTMA (1954, table 22 and fig. 33) illustrate the possibility that the quantity of chlorophyll decreases to less than half as reckoned from the tidal inlet proper, while the quantity of suspended material increases over the same distance, by a multiple sometimes reaching a value of 50–100 mg silt per litre.

Now in the case of oysters, LOOSANOFF and TOMMERS' observations (1948) have shown that, when the quantity of anorganic silt is raised, then the quantity of water which these oysters allow to pass through their gills falls sharply. At concentrations of silt between 100 and 400 mg per litre the quantity of water which they allow to pass through fell by, respectively, 50 to 90 per cent. The mussel, too, shows the same propensity (TAMMES and DRAL 1955). The assumption that the same also applies to the cockle readily suggests itself. On the supposition that, in silt-free water, it is the need for food, and not for oxygen, that determines the intensity with which the cockles pump the water through their gills, any weakening of this pumping activity will adversely affect the nutritional condition of the cockles. We have made several attempts to determine with certainty the effect of material suspended in the water on the growth-rate of cockles; but owing to technical difficulties these efforts have so far been unsuccessful. Since we attach essential importance to the possibility that suspended material may have an inhibiting action on growth, we intend to continue our endeavours in this direction.

9. *Influence of the available quantity of food*

Many authors who have investigated the growth-rate of *Cardium* have demonstrated that cockles grow fast in places where currents are strong and where the submersion-time is long, but that stunted growth is observed wherever the water stagnates and the submersion-time is short. None of these authors expects that the current velocity or the duration of submersion, as such, could have any influence in this respect; they assume that these two factors are determinants only of the quantity of food which the cockles are able to seize (STEPHEN 1932, p. 65; THAMDRUP 1935, p. 106; KREGER 1940, p. 192; LINKE 1940, p. 276; SMIDT 1952, p. 93). We ourselves are also convinced that the submersion-time may be so short that the cockle has insufficient time to take in food. On the other hand, we do not believe that the quantity of food available to the cockle is determined to any important extent by the current velocity, as we shall explain below.

We shall examine the possibilities of food deficiency as a result of (a a weak current, (b short submersion period, and (c shortage of food in the water itself which latter might be due to previous filtration of the water by other plankton feeders (i.e. competition).

When inquiring into the influence of the available quantity of food on the growth-rate it should be constantly born in mind that, although the water may contain an abundance of suitable food, its intake by the cockle may be hampered by transported or suspended material, as discussed in the preceding paragraphs.

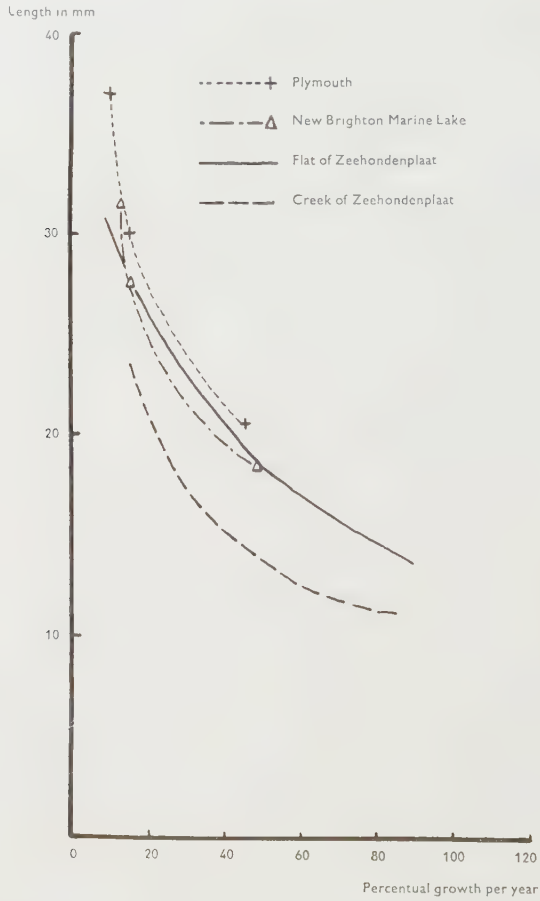

Fig. 25. Percentual increase in length per year, of cockles: a. from the vicinity of Plymouth (according to ORTON's data, 1926); b. from a sea-locked marine lake near New Brighton (according to ORTON and co-workers' data, 1936 and 1937); c. and d. of the flat and of the creek of the Zeehondenplaat.

a. Current velocity and quantity of food

ORTON and co-workers (1936 and 1937) give some figures about the growth of cockles in a small salt-water lake completely shut off from the sea, on the west coast of England. The growth-rate here was only a little below that of the rapid growth of cockles from the neighbourhood of Plymouth (ORTON 1926), and it was equal, for instance, to that on the top of the Zeehondenplaat (see fig. 25). This proves that a strong current, as such, is not an absolute condition for the prosperous growth of cockles.

That the growth-rate of cockles on a flat does not always run parallel with the velocity of the current may also be deduced from ORTON's experiments (1926) with cockles in a practically closed-in box, placed on the flat. Although the current flowed at a very slow rate through the small holes in the box the cockles in this box grew as fast as those on the open flat.

All the same, there are not many exceptions to the rule that the cockles' growth increases parallel with the average current velocity. True, on the Zeehondenplaat we could not demonstrate any differences in current velocity corresponding to the differences in growth as between the high- and the low-lying parts; but the current was here measured only at the surface. The fact that so much detritus was deposited on the low part (i.e. in the creek) makes quite obvious that the current velocity, there at the bottom, was lower than at the top of the flat.

Could it be that a rapid current brings along a larger quantity of food than a weak one? In fact, more food will pass by the cockle per unit of time according as the current velocity increases. But the cockle's pumping velocity is so slight (0.5 litre per hour, cf. WILLEMSEN 1952) that a low current velocity of a few cm per minute should be sufficient to assure the cockle of a constant supply of fresh water. (The situation changes of course in a *dense* cockle population, where mutual competition might play a role, vide p. 440).

The weakest currents we ever measured on any flat had none the less always a velocity of some tens of cm per minute, even om a high-lying flat closed in by small dykes on two sides, such as the one found at the Balgzand. Here the velocity of the current, on the highest point where cockles are still found, was about 60 cm or more per minute from the moment of submersion.

But the velocity of the current might affect the growth of cockles in yet another way. It is conceivable that with the increase of the current velocity the quantity of food that passes the cockle not only increases per unit of time, but also per cm^3. This would then apply to the quantity

of bottom diatoms and detritus. But these diatoms occur most numerously not outside the estuary, but far into the Wadden Sea (cf. GRØNTVED 1949, p. 38). It accordingly seems improbable that the explanation why cockles grow faster in a strong current than in a weak one should have to be sought in the measure in which diatoms are swirled up from the bottom. Neither is it conceivable that the quantity of whirled up detritus can have much influence, because detritus, too, is not found so much outside the tidal inlet as in the Wadden Sea (POSTMA 1954). On the contrary, what seems more probable is that it is precisely the large quantity of detritus in the Wadden Sea that inhibits the cockles' growth there.

One wonders what, then, could be the reason why, in most places, a strong current is coupled with a fast rate of growth in cockles, and a weak current with a slow rate. One might readily suspect this phenomenon to be a result of competition for food: in a dense population more food will become available according as the current is stronger, so that the animals will grow faster. As we shall see below, however, it is a rare exception for cockle populations to be so dense that there can be any question of competition for food.

As we have mentioned before we suspect that the current velocity influences the rate of growth in a different way. In places where the current is weak more silt and detritus settles than in places with a stronger current; and we regard the settling of substances suspended in the water as the most important factor hampering the cockle's intake of food.

Our conclusion is therefore that the current velocity as such hardly affects the quantity of food taken in by the cockle. That the cockle generally grows faster according as the current is stronger is caused, in our view, by the fact that the fast current prevents the sedimentation of suspended substances.

b. Duration of submersion and quantity of food

As HAZELHOFF (1937, p. 325) remarks, the normal quantity of water which a suspension feeder such as *Cardium* pumps through its gills is not related to the animal's normal need of oxygen, but to its normal need of food. Now as the submersion-time of the flat is shorter the cockle will pump less water; and it is therefore quite understandable that the growth-rate of the cockle is affected by the length of the submersion period.

As we have seen, a short period of emergence does not affect growth, providing this period does not last much longer than 6–7 hours per tide. On the Zeehondenplaat, which emerges for about three hours, the

animals are evidently able to collect sufficient food during the remaining nine hours, so that they do not, as regards growth, lag behind other animals that can collect food continuously.

One might readily suspect that *young* cockles (say, of 2 mm), because of their great relative rate of growth, would sooner suffer from a short submersion-time than older ones. Perhaps this may be so. Our investigation on the Zeehondenplaat shows that the cockle spat around low-water level and on the top of the flat *simultaneously* reach a length of 7 mm (see fig. 8, p. 373), while it appears from their growth above 7 mm that conditions for growth are definitely more favourable on the top of the flat. The animals of a few mm length, therefore, have not been able to profit by these more favourable circumstances on the higher part—maybe precisely on account of the period of emergence of some hours per tide, which is too long for them. We have, however, no data that might throw light on this; the more so as, during emergence, abiotic factors can play a role, particularly as regards the very smallest cockles (cf. p. 427).

c. Shortage of food in the water

When the water contains too little food this may be because there is a general lack of plankton suitable for the cockle's nourishment; but it may also be caused by the local presence of so many feeders on plankton together that their competition causes the "wash to be poor". It is unfortunately still an open question what part of the plankton is consumed by suspension feeders such as *Cardium*. Undoubtedly nannoplankton organisms such as some species of flagellates and small diatoms have their importance; but maybe other plankton organisms or suspended detritus can be digested (see survey by VERWEY 1952, p. 207–212).

So long as we do not know which organisms are, and which are not, accepted as food by the cockle, we cannot judge the influence which seasonal fluctuations in the composition of the plankton could possibly exercise on the cockles' rate of growth. Assuming that cockles are more or less specialized on feeding on flagellates, then the dangers of a temporary food deficiency are naturally greater than if they consume also other phytoplankton organisms (diatoms!) and detritus.

A food deficiency might arise when the population reaches such a density that the food content of the water locally becomes exhausted. When the water is stagnant it is conceivable that each cockle keeps pumping in its own pumped-out water again, and that of its neighbours. It is possible with coloured water to demonstrate that this is only partly correct; for the water, although sucked in mainly from the animal's close vicinity, is subsequently for the greater part spouted up again further

than 10 cm away. In spite of this the cockle, in the absence of current, will chiefly exploit only the water in its immediate vicinity, i.e. the bottom water. Stronger turbulence and greater supply of water not yet completely filtered, therefore, are necessary conditions for a prosperous growth of the cockles. The degree of competition for food accordingly does not depend solely on the density of the population, but also on the expanse covered by this population, plus the velocity of the water-current, plus—be it to a lesser degree—on the level of the water. Since the extent of the population and the current velocity during submersion are sufficiently known of hardly any flat at all it is in most cases extraordinarily difficult to determine the role of the competition factor. In fact, we know of only one or two cases where the influence of competition can be proved.

Thus, in one case it was found that the presence of *mussels* inhibited the growth of cockles. This was on the Amelander Slik. In 1947 there were no mussel-banks here at first. In the summer of that year a cockle population settled there, reaching in the first year, as shown by their first winter-ring, a size of 14 mm. Meanwhile, during the autumn of 1947 and the spring of 1948, an extensive set of mussel-banks developed there as well. In 1948 and 1949 the cockles that were lying away from these mussel-banks grew successively by 6 and 4 mm, while the cockles lying on the lee-side of the mussel-banks grew only by 3.5 and 2 mm respectively, i.e. less by almost half.

A similar example of mussels inhibiting the growth of cockles we have mentioned already earlier, see p. 416.

The assumption is obvious that the *cockles* also compete *with each other*. It is, in fact, remarkable that in places where the density is low the cockles nearly always grow fast. At a high population density, on the contrary, growth is almost invariably slow. This phenomenon is found both on the Scottish cockle banks (Stephen 1931) and on the Wadden Sea banks. In a few places (Zeehondenplaat, Staart van Schieringhals, Paardenhoek) any increase in density is even coupled with a decrease in the length of the cockles, so that we were at first convinced that there existed some causal connexion between the two, in the form of competition for food (Kristensen 1953, p. 56). On the Zeehondenplaat, for instance, the population in the creek was in 1947 ten times denser than on the top of the flat, while the linear growth in the creek was about 30 per cent. below that on the flat. If, however, there had been any question of competition for food one might have expected that, in the years when the population would be less dense, the difference in the rate of growth would also be correspondingly smaller. But this turned out not to be the case; for four years later, when not much was left of the year-class 1947, a fresh spat-fall was

reported to have settled in the same area. This year-class 1951 was not half as numerous as the year-class 1947 had been; but the growth of the year-class 1951 was at first practically equal to that of 1947 (see table XXXII). The supposition that a stronger influence of the competition factor in 1947 had been compensated in that year by more favourable conditions for growth (warm summer) is at once refuted by the fact that, in that case, the cockles on the top of the flat should have grown faster than they did in 1951—which was not the case.

TABLE XXXII

Length and density of the cockle year-classes 1947 and 1951 on the Zeehondenplaat, in the creek (No. 5) and on the flat (Nos. 10–12) (Figures in parentheses after the length represent the standard deviations, n, in mm).

	1st winter-ring in mm (n)	density per m^2	2nd winter-ring in mm (n)	density per m^2
Creek-cockles:				
Year-class 1947	13.3 (1.3)	3500	20.2 (1.2)	1000
Year-class 1951	13.2 (1.4)	1400	23.8 (1.2)	495
Flat-cockles:				
Year-class 1947	18.1 (2.6)	200	28.3 (2.0)	145
Year-class 1951	18.0 (2.5)	110	28.5 (2.1)	14

We see therefore that there is no noticeable competition for food among a cockle population with a density of 3.500 specimens per m^2, at any rate not during the first year. As table XXXII shows, the population of the 1951 year-class in the creek grew distinctly faster, during the second year, than the 1947 year-class. Probably, however, this is no attributable to the competition factor either, since the morphology of the creek changed rather considerably in 1952: we estimated that the difference in level between the creek and the flat was reduced by about half, while the difference in average grain size of the sand fell from 22 per cent. to 8 per cent. (see fig. 6, p. 364). Although the change in grain size does not in itself affect the growth-rate of the cockles, the layer of detritus formerly deposited there now and then (cf. p. 432), also disappeared to a large extent.

From fig. 14 (p. 407) one might conclude that the growth-rate of the cockle year-class 1947 in the creek in its first summer was distinctly below that in the first summer of year-class 1951. This would seem to be in contradiction to what is stated above in considering table XXXII. This discrepancy, however, is only apparent, being due to the fact that in fig. 14 the average of *all* creek-cockles has been taken to represent the growth of the creek-cockles of the 1947 year-class, while for year-class 1951 we only had sufficient data of sample 5 at our disposal. The growth-rate of the cockles at point 5, however, is slightly faster than at points 3 and 4. In table XXXII only details from a single sampling place (No. 5) have been elaborated with respect to the creek.

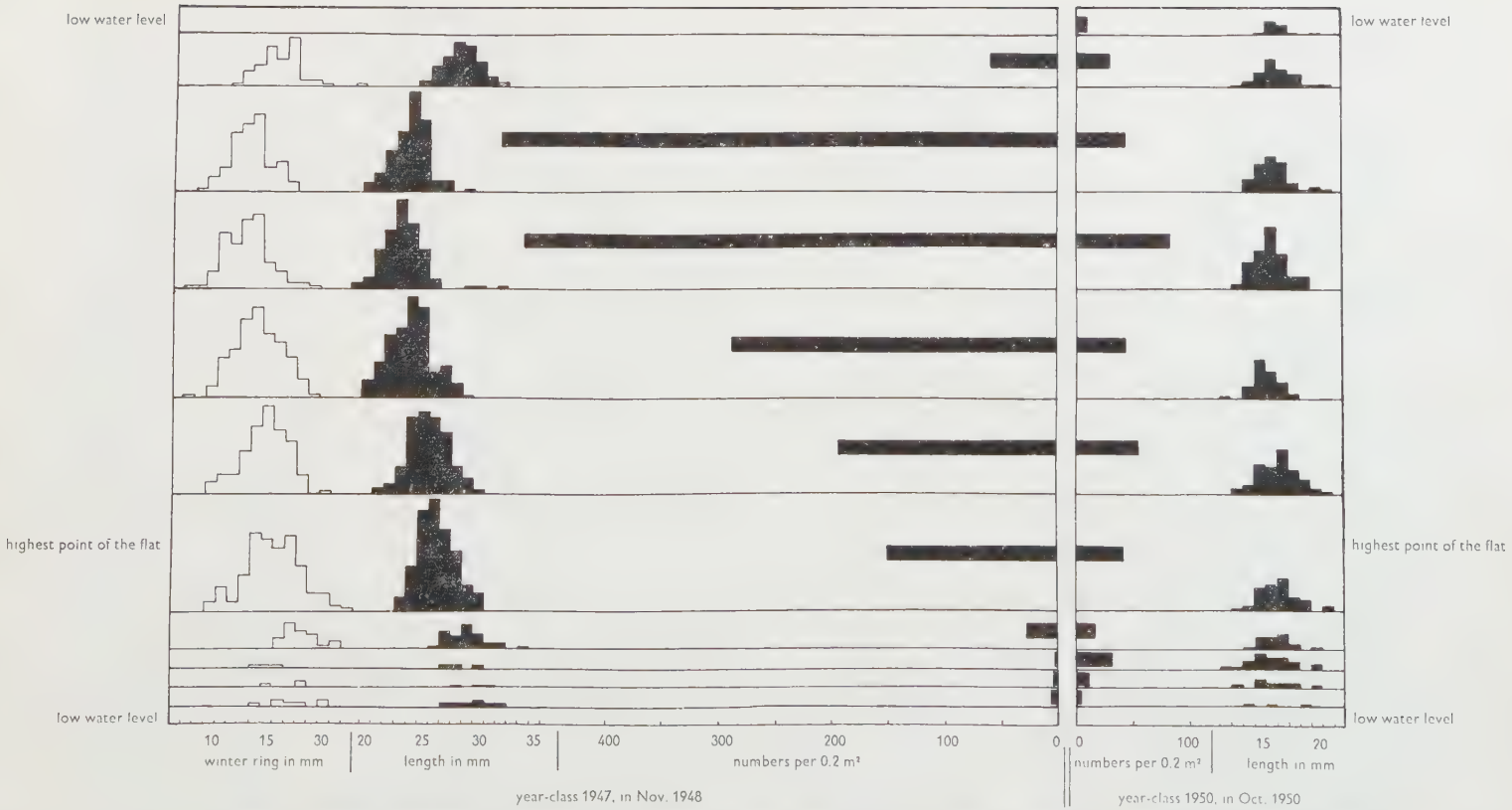

Fig. 26. Black squares: diagrams of density and length of the cockles of the year-classes 1947 and 1950 on the Zuidwal.
Blank squares: length of the first winter-ring of the cockle year-class 1947. (The two largest samples of the year-class 1947 were only partly measured.)

To find a place elsewhere in the area investigated, where the competition factor did have an influence on the growth-rate we must look for a flat where the ratio *population density* × *extent* : *current velocity* is more unfavourable for the cockles' growth than in the creek of the Zeehondenplaat. Everywhere else, however, the population density is so much lower that one can assume that the influence of the competition factor can have been of significance practically nowhere. We know of only a single exception to this, viz. at the east point of the Zuidwal (see fig. 26). Both in the first and in the second year of the cockle year-class 1947 there was a relation in this locality between the growth of the cockles and their number. The greatest density of the ± 18-month-old cockles was here about 2000 specimens per m^2. At this density the size of the cockles was about 15–20 per cent. less than in other places. Two years later this flat was examined again; the 1947 year-class had to a large extent disappeared by then, and a new spat had settled. This spat, which was far less numerous than the 1947 year-class previously, grew up perfectly uniformly, without the flat giving the impression of having altered in the meantime. We assume therefore that competion for food had, in fact, influenced the growth of the dense population of the 1947 year-class, both in the first and in the second year of its life. Unfortunately, it is not known how dense the population was in the first year. In the second year it was—as mentioned above—± 2000 per m^2.

From KREGER's figures (1940) relating to the density and the growth of the cockle population of this same Zuidwal flat one gathers the impression that, also at that time, the density was so great locally, that the competiton for food affected growth; but no proof of this can be given because no years of great and of small population density can be compared with each other. Unfortunately no further investigation could be made on this splendid flat, because it disappeared owing to the extension of the Naval Port Den Helder.

Our conclusion is that the differences in growth on the Zeehondenplaat have not been caused by competition, but by abiotic factors (very likely detritus), while on the Zuidwal the differences in growth were probably the result of competition. On a flat with an average submersion-time of ±5½ hours per tide, at an average current velocity of ± 7 m per minute, and a density of ± 2000 one-year-old cockles per m^2, it is possible that competition for food causes the length of these cockles to be about 15–20 per cent. less than that of a smaller population under the same conditions.

VI. GENERAL REMARKS

Our investigation having enabled us to obtain some insight into the demands which the cockle makes on its environment in the area we have

studied, one might expect that it would not be difficult to give a description of a milieu which would be an optimal one for the cockle,—if not in all respects, at any rate in most. We have seen that in exposed places growth is rapid and mortality low. As against this there is the fact that in such places the spat has great difficulty in settling, in addition to which it is very much exposed, at any rate during the first few weeks of life, to the danger of being washed away. The result of this is that, as a rule, the population in exposed places is not dense, a thing which will adversely affect the total egg production of the species. This raises the question where then to look for the cockle's ecological optimum.

The concept "optimum", as used in ecology, is derived from physiology; there, conditions are called optimal when they enable the process investigated to follow the most complete and rapid course. In ecology, in which not part of an organism, but an entire animal, or even a number of animals constitute the subject of investigation, it is necessary to determine the optimal conditions for each separate relation which such a "unit" maintains with respect to its environment.

Now on the assumption that the animal and its habitat together form a harmonious whole, it would appear possible to form, on the ground of ecological investigations, a mental picture of the animal's ecological optimum. But this is possible only so long as no opposing interests assert themselves in the different ecological relations. For, as soon as the interests of one relation are opposed to those of another, we should have to draw up a hierarchy of important and less important relations, if we are to form an idea of what should be the ecological optimum.

Among the most important relations between the species *Cardium edule* and its habitat we may reckon those that can be deduced from the rate of growth and the chances of life of the individual.

As regards the *rate of growth*, this is, for cockles > 6 mm, highest in exposed places; but this—as we have seen—probably does not apply to smaller cockles.

With regard to the animal's *chances of life*, these are, for settling spat, favourable in sheltered places; later on, however, the chances of life are most favourable in exposed places.

But when the individual's chances of life are *not* optimal in a given place, that does not mean that this is a disavantage to the existence of the species. For instance, if during a severe frost, all individuals perish on the emerging flats, then the very condition has been created for the settlement of a new population that is denser than the preceding one.

Finally, for the species *Cardium edule* to maintain itself one relation, not so far discussed, is of great importance; viz. the relation which may be deduced from the *egg production* per area-unit of the region investigated.

In order to get some idea of the quantities of eggs produced on the various parts of a flat, we first determined the egg-production of animals of different sizes. To this end a number of cockles of different ages and sizes (12–31 mm) were collected on the Zuidwal in April 1951, and the number of ripe eggs of these animals determined. By means of interpolation it was then calculated how many eggs the cockles on the Zeehondenplaat would have possessed in the years following 1947.

Since the conditions for growth were so bad in the creek of the Zeehondenplaat, we could imagine that the cockles there would produce fewer eggs than equally large animals elsewhere. On the ground of an observation made in June, 1952, however, this is improbable. We then examined a few equally large animals from the flat and from the creek, when it was found on a rough count that the number of ripe eggs was the same in both flat- and creek-cockles (i.e. ± 3000 per cockle of 26 mm). On the assumption that cockles of the same size but from different environment produce the same number of eggs we may now draw up table XXXIII:

TABLE XXXIII

Computation of the quantities of eggs produced annually in April by the cockles of the year-class 1947 on the flat and in the creek of the Zeehondenplaat

	1948	1949	1950	1951	1952	1953
On the flat						
Length of the cockle in mm	19.4	28.1	32.0	35	37	38
Numbers of ova per cockle	5600	20000	31000	44000	48000	52000
Numbers of cockles per m²	150	60	35	20	15	6
Numbers of ova per m²	0.8 mill.	1.2 mill.	1.1 mill.	0.9 mill.	0.7 mill.	0.3 mill.
Total number of ova per m² *on the flat* from 1948–1953: **5.0 million**						

	1948	1949	1950	1951	1952	1953
In the creek						
Length of the cockle in mm	14.0	20.6	25.6	29	35	—
Numbers of ova per cockle	2900	7800	16500	21000	44000	—
Numbers of cockles per m²	1000	300	200	50	20	0
Numbers of ova per m²	2.9 mill.	2.3 mill.	3.3 mill.	1.0 mill.	0.9 mill.	0
Total number of ova per m² *in the creek* from 1948–1953: **10.4 million**						

According to our calculations the production of eggs per unit area on the Zeehondenplaat would have been about twice as high in the sheltered creek as on the exposed part of the flat, despite the fact that the cockles lying exposed have grown faster and show a lower mortality than those in the creek. In an area like the North Sea, where cockles grow very fast, the average density of the population is so low that the production of eggs per m² is sure to be very small there.

Now if one wished to determine the ecological optimum of the cockle,

on the basis of the above mentioned relations, one would arrive at a different result, whether the small settling spat or the older individual were considered, or whether the individual or the species were considered.

One might perhaps imagine that this did not apply to another, more optimally situated part of the cockle area – say, somewhere outside the Wadden Sea region. We ourselves are not aware of the existence of such an optimal area. On the south coast of England, ORTON (1936) observed a rate of growth slightly higher than that in the Wadden Sea; but the population density was no higher there than it is here. No denser populations than are found locally in the Wadden Sea are known either in Britain or anywhere else in western Europe. And nowhere does one find both a high rate of growth *and* a high population density in one and the same place.

In this, the cockle probably does not stand alone. We find a similar situation in the case of both *Mya arenaria* and *Mytilus edulis*. It is probable that this lack of an ecological optimum is inherent in every sedentary euryokous species. It is surely not a matter of accident that also the larvae of these species show considerable tolerance towards environmental differences, and only slight preference for one particular milieu.

In *non*- sedentary euryokous species this situation is probably not very different. For a species like the plaice *(Pleuronectes platessa)*, for instance, the ecological optimum is definitely not in brackish water such as that of the Baltic; for the plaice in its first year of life grows more slowly there than in a non-brackish area such as the North Sea; neither does it reach in later years the size of the plaice in the North Sea. None the less, the relative growth of the *older* plaice is in the Baltic no worse than in the North Sea; on the contrary; older plaice that are transported from the North Sea to the Baltic are found to grow faster in the latter than in the former. This means that, in some respects, the Baltic may even be a more optimal milieu for plaice than the North Sea.

And the experiments of KINNE (1956, p. 8–9) show that brack-water species like *Cordylophora caspia* and *Gammarus duebeni* seem to have an optimum salinity that is much higher than the salinity found in their normal habitat.

In non-sedentary animals, moreover, one also finds the phenomenon that the very young animals show relatively little preference as regards their milieu. Young *Crangon crangon*, *Carcinus maenas*, *Portunus holsatus*, *Clupea harengus*, *Gadus merlangus*, *Pleuronectes platessa*, etc. populate a larger area than the adult animals. Since the animals, as they get older, leave the boundary areas of their region, their preferential area evidently becomes gradually more narrowly delimited. (It is naturally im-

possible to investigate the extent to which the preferendum of a *sedentary* species may undergo any change.)

For stenokous species the situation is surely altogether different. As many investigations of recent years have shown (cf. WILSON 1952) the larvae of species which are stenokous in respect of the environmental factors involved have a strong preference for settlement in an area that satisfies quite well-defined demands. Some species settle, during certain years, outside their normal area, when circumstances cause the environmental factors in the new area to be favourable for settlement. In 1947 we saw how, in a period of high salinity, a few stenohaline species, as *Cardium crassum*, *Dosinia exoleta*, *Pecten maximus*, *Nephrops norvegicus*, *Onos cimbrius* and *Hippoglossoides platessoides* settled in the southern part of the North Sea. During the same summer *Astropecten irregularis* and *Ophiura texturata* settled as far as the inner parts of the Wadden Sea, where they are not usually found under normal conditions. Although the change of salinity in 1947 was only of a temporary nature, the species mentioned were found to have maintained themselves in the newly populated areas during the next few years (but they disappeared again for the most part later on, because the populations were not being supplemented by new spat).

Although, therefore, the area of tolerance of rather stenokous species may evidently be larger than would be expected from their normal distribution, we believe that one might be quite justified in speaking of an ecological optimum with respect to these species; at any rate, it should be possible to determine approximately the milieu with which a stenokous species "fits in well". So it is quite possible to describe the optimal milieu of, e.g., corals, parasites and other typically stenokous species. For euryokous species—particularly the sessile ones among them—this is quite a different matter. There is no milieu to be found in which they fit in all respects. Are such euryokous species, therefore, "badly adapted", or "badly fitted in"? If one likes: yes, providing one bears in mind that it is precisely in this "poor fit" that resides their strength. The word "bad" or "good", in fact, is out of place in connexion with the concept "fitted in". Ecological "fitting in" should be regarded as a form of specialization; the euryokous species shows a weaker specialization of form and function than a stenokous species. The various optima which can be demonstrated in a typically stenokous species in its relations with respect to its environment will, first of all, be sharply delimited, and naturally, therefore, will have to coincide. The optima of a euryokous species, on the contrary, are not sharply delimited, and often will not coincide.[1]

[1] This holds especially for species which are stenokous *in sensu stricto;* we hope to come back on the problem of the different forms of stenoky in a subsequent publication.

An animal species such as the cockle, therefore, will never find an optimal milieu anywhere in its area of distribution; always and everywhere the situation will represent a compromise. The idea of an "ecological optimum" here is a wish-dream—not on the part of the cockle, but of the biologist.

VII. SUMMARY

After the severe winter of 1946-'47 the tidal flats of the Dutch Wadden Sea were completely clear of cockles. In June 1947 there followed an exceptionally rich spat-fall, while the spat that fell during the next few years was comparatively poor. This state of things provided an opportunity of investigating the local differences in growth and density, of the year-class 1947, in the course of a number of years.

It was found that the settlement of the spat was contingent in the first place upon the degree of protection which the tidal flat offers against the transporting action of the current; but on those parts of the flat which emerge for a period exceeding 6-8 hours per tide, the lasting settlement of spat is also influenced by the fact that the time allowed for the intake of food is too short. In the deeper channels of the Wadden Sea the spat does not settle definitely anywhere, not even in the sheltered places. This is probably because too large a quantity of detritus and silt is deposited on the bottom whenever the water is temporarily stagnant. There is also the probability that a very dense population of old cockles inhales so much spat—i.e. spat smaller than 1 mm—, that this affects the primary settlement of the spat, but since the slightly larger spat is too big to be inhaled, but not yet too big to be transported, it will eventually settle among the adult cockles, so that the effect of even very dense populations has only little influence on the final settlement.

In places favourable to the animals' growth the annual diminution of a population is found to be between 10 and 45 per cent. Under unfavourable conditions this percentage may rise to 70 or even higher. Moreover, where the cockles grow faster, the maximal duration of life is longer than in places where they grow less rapidly. In the western part of the Wadden Sea the maximal duration of life of a cockle is at least 8 years.

A decrease in the density of the population as a result of its being washed away occurs in the case of very small spat (<2 mm), which is as yet unable to dig itself in deep enough; but, in addition, cockles of 3 years and older, too, are often washed out, because the digging-in speed decreases as the animals get older. Because digging-in proceeds many times more slowly in winter, at low temperatures, than at summer

temperatures, the cockles are most exposed in winter to the danger of being washed out and transported.

Especially in severe winters the mortality can be very high; the cockles then perish not only as a result of being washed away and transported to unfavourable locations, but also as a direct result of being frozen. Finally they can die due to the tidal flat being covered with a sheet of ice below which hydrogen sulphide is formed.

Many cockles also die in springtime. It was found improbable that this could be due exclusively to the widely fluctuating water temperatures to which animals living on tidal flats are exposed particularly in the spring; this mortality probably involves animals that have been weakened during the preceding winter, and in which the fatal process has merely been accelerated by the changing temperatures of the water.

The clearer the water the deeper the cockles are found below low-water level. This is not because the animals are more likely to die in deeper water due to lack of light, or that they might be an easier prey in deeper water because they do not dig themselves in either so quickly or so deep. But what does seem probable is that, when predators approach in the dark, a cockle reacts less promptly by closing its shell, a circumstance which is bound to have an adverse effect on the population density.

No clarifying data were obtained concerning the possible effect of the structure of the bottom and of detritus suspended in the water, on the mortality.

The fact that the presence of even a small number of older cockles causes the mortality among one-year-old cockles to be higher than in places where there are no older cockles, points to the possibility that infection by parasites raises the mortality. Such parasites, however, could not be identified.

Birds may locally decimate the population on the tidal flats. Fish appear to feed on young cockles, especially below low-water level.

Investigation into the *growth* of cockles showed that the animals increase in length, in their first year of life, until the end of September; further, that this growth in length is halted during winter, and that during succeeding years the period of increase in length is limited to the spring and early summer (viz. from the end of March to July). The relative rate of growth is, mathematically, a function of the animal's length—not of its age. In places of poor growth this function invariably has a lower value than in places where the cockles grow fast—at any rate when comparing animals of equal length. The relative rate of growth diminishes with increase in length; but obviously the rate of growth of a given length-category is not the same every year.

In investigating the local differences in growth we used the length of

the shell as a measure of growth, although it was found that increase in length does not invariably run parallel with increase in weight. In places where growth is poor the cockles' weight may be relatively low, in addition to which changes in the ratio length : weight occur in the course of the season. After a period of rapid increase in length the shell is, relatively, very light—that is, at the end of the first summer of the animal's life, and again after the period of growth in the early summer of the second year. The relative weight of the flesh decreases slightly during the winter and increases again in spring, notwithstanding a rapid increase in the length of the shell, and the discharge of sexual products at that time.

It is shown that the differences in length between cockles of the same year-class and from the same spot cannot be explained by assuming that the larger ones are a couple of weeks older than the smaller ones. Neither can these differences be due to congenital factors, nor can they be caused by the frequency with which the cockles are washed out and transported. The differences in length are supposed to be caused mainly by local differences in the environment.

It was found improbable that there is any influence of the salinity on the rate of growth in the area studied. Neither could any effect of the temperature, or of light, be demonstrated as the cause of the local differences in size. Furthermore, it appears improbable that the available quantity of oxygen could be a factor in causing the differences in size that were observed.

The structure of the bottom does not influence the growth-rate of the cockles; but the layer of detritus which may pass over the flat with the movement of the tide does, it is believed, adversely affects growth. In addition, moderately large quantities of material suspended in the water seem to be harmful to growth—probably because any fairly large quantity of particles in the water retards the cockle's pumping speed. The current velocity is important in this, because it affects the concentration of particles in the water. The view that the velocity of the current could have any considerable influence on the quantity of food taken in by the cockle has been found erroneous.

When the period of submersion is shorter than $\pm$ 5 hours per tide, growth will be affected, because the cockle evidently needs at least 5 hours per tide to gather enough food.

The cockles' growth may further be adversely affected by the presence of mussels—no doubt because of lack of food.

Competition among cockles arises only when both the density and the extent of the population are considerable while, at the same time, the current velocity is low. Careful tests, in which two year-classes of different density on the same tidal flat were successively compared,

showed that the effect of competition for food does not manifest itself nearly so promptly as we had suspected. It is, in fact, conspicuous by its rarity.

What has become clearly evident from our investigation is that there are locations in this area that favour the settlement of spat, which gives rise to a dense population with a great production of eggs per unit area. In such places the growth in length of individual cockles—at any rate from the moment they reach a length of a few mm—is distinctly poor; the mortality among the cockles is high, and their maximal length of life is short.

In other places, on the contrary, where only little spat can settle, growth is rapid, mortality low, and the maximal duration of life comparatively long,—but the quantity of eggs per unit-area, because of the low population density—is small.

In attempting to determine an ecological optimum for an animal such as the cockle one meets with difficulties of a treble nature: first, the various relationships existing between the cockle and its environment do not always have their optimum in common; secondly, there may be a shift in the optimum in the course of the individual's life and thirdly, what is optimal for the individual may be very far from optimal for the species, and vice-versa. To this absence of a well defined ecological optimum for the metamorphosed animal must be added the absence of a pronounced preference on the part of the larvae during settlement. This is considered to be typical of euryokous, sessile animals. Absence of an ecological optimum is also typical of euryokous, *non*-sessile animals; but these animals' preferences are narrowed down and modified as they get older.

Typically stenokous animals, on the contrary, have a pronounced preference for a certain milieu as long as they live; and this milieu roughly corresponds to their ecological optimum.

The terms "good" or "poor" fitness are wrong when applied to stenokous and euryokous animals respectively; euryokous species merely show a lesser degree of specialization of form and function with respect to their milieu than stenokous species.

VIII LITERATURE

BAGGERMAN, B., 1953: Spatfall and transport of *Cardium edule* L. Arch. Néerl. de Zool., **10**, 315–342.

BIRSTEIN, J. A., 1936: The growth and propagation of *Cardium edule* of Mertvy Kultuk and Kaidak in Caspian Sea in connection with salinity. C.R. (Doklady) Acad. Sci. U.R.S.S., **4**, 195–199.

BLEGVAD, H., 1917: On the food of fish in the Danish waters within the Skaw. Rep. Dan. Biol. Stat., **24**, 17–72.

BRAUN, R., 1954: Zum Lichtsinn augenloser Muscheln. Zool. Jahrb. Abt. Allg. Zool. Physiol. Tiere, **65,** 194–208.

BREEMEN, P. J. v. and REDEKE, H. C., 1907: Bijdrage tot de kennis van de flora en fauna der Zuiderzee. Zuiderzeerapport, Bijlage v, 1–21.

DAM, L. v., 1935: On the utilization of oxygen by *Mya arenaria*. Journ. Exp. Biol., **12,** 86–94.

HAVINGA, B., 1922: Mariene Mollusken. Flora en Fauna der Zuiderzee, 373–390.

HAVINGA, B., 1928: The daily rate of growth of oysters during summer. Journ. du Conseil, **3,** 231–245.

HAVINGA, B., 1929: Krebse und Weichtiere. Handb. d. Seefischerei Nordeuropas, Band **3,** Heft 2, 1–147.

HAZELHOFF, E. H., 1938: Über die Ausnutzung des Sauerstoffs bei verschiedenen Wassertieren. Z. vergl. Physiol., **26,** 306–327.

JOHNSTONE, J., 1899: *Cardium*. Memoirs Liverpool Mar. Biol. Comm., **2**.

KAMPS, L. F., 1950: Enige gegevens over de sedimentatie in het Waddengebied ten noorden van de provincie Groningen. Waddensymposium. Tijdschr. Kon. Nederl. Aardrijksk. Genootsch., **67,** 109–113.

KINNE, O., 1956: Über den Wert kombinierter Untersuchungen (im Biotop und im Zuchtversuch) für die ökologische Analyse. Naturw., **43,** 8–9.

KORRINGA, P., 1940: Rapport over het onderzoek naar den broedval van de oester. Versl. en Meded. Afd. Visscherijen No. 35, den Haag.

KORRINGA, P., 1949: More light upon the problem of the oyster's nutrition? Bijdragen tot de Dierkunde, **28,** 237–248.

KREGER, D., 1940: On the ecology of *Cardium edule* L. Arch. Néerl. de Zool., **4,** 157–200.

KRISTENSEN, I., 1953: De oecologie van de kokkel (*Cardium edule*). Geloof en Wetenschap, **51,** 54–56.

LARSEN, K., 1936: The distribution of the Invertebrates in the Dybsø Fjord, their biology and their importance as fish food. Rep. Dan. Biol. Stat., **41,** 3–35.

LINKE, O., 1939: Die Biota des Jadebusenwattes. Helg. Wiss. Meeresunters., **1,** 201–348.

LOOSANOFF, V. L. and F. D. TOMMERS, 1948: Effect of suspended silt and other substances on rate of feeding of oysters. Science, **107,** 69–70.

MACKIN, J. G., P. KORRINGA and S. A. HOPKINS, 1952: Hexamitiasis of *Ostrea edulis* L. and *Crassostrea virginica* (Gmélin). Bull. Mar. Sci. Gulf and Caribbean, **1,** 266–277.

ORTON, J. H., 1926: On the rate of growth of *Cardium edule*. Part I. Experimental observations. Journ. Mar. Biol. Ass., **14,** 239–279.

ORTON, J. H., 1933: Summermortality of cockles on some Lancashire and Cheshire Dee Beds. Nature, **132,** 314–315.

ORTON, J. H., 1934: Bionomical studies on *Cardium edule*. With special reference to mortality in 1933. James Johnstone Memorial Volume, Liverpool, 97–120.

ORTON, J. H., c.s., 1936: Rate of growth of *Cardium edule*. Nature, **137,** 705–706.

ORTON, J. H., c.s., 1937: Rate of growth of totally submerged *Cardium edule*. Nature, **139,** 799–800.

POSTMA, H., 1954: Hydrography of the Dutch Waddensea. Arch. Néerl. de Zool., **10,** 405–511.

POSTMA, H., 1957: Size frequency distribution of sands in the Dutch Wadden Sea. Arch. Néerl. de Zool., **12,** 319–349.

POSTMA, H. and J. VERWEY, 1950: Resultaten van hydrografisch onderzoek in de Waddenzee. Waddensymposium. Tijdschr. Kon. Nederl. Aardrijksk. Genootsch., **67,** 3–33.

PURCHON, R. D., 1939: The effect of the environment upon the shell of *Cardium edule*. Proc. Malacol. Soc. London, **23,** 256–267.

QUAYLE, D. B., 1952: Structure and biology of the larva and spat of *Venerupis pullastra* (Montagu). Trans. Roy. Soc. Edinb., **62,** 255–297.

REDEKE, H. C., 1906: Verslag omtrent onderzoekingen over het voedsel van eenige visschen. Jaarboek Rijksinstituut Onderzoek der Zee, 1905, 88–111.

REDEKE, H. C., 1914: Bijdragen tot de teelt van de ansjovis in de Zuiderzee. 2e stuk. Rapp. Verh. Rijksinst. Visscherij-onderzoek, **1,** 241–266.

REMANE, A., 1934: Die Brackwasserfauna (Mit besonderer Berücksichtigung der Ostsee). Verh. Deutsch. Zool. Ges. E.V., 34–74.

RINGER, W. E., 1906: Über die Veränderungen in der Zusammensetzung des Meereswassersalzes beim Ausfrieren. Verhand. Rijksinst. Onderz. Zee, **1,** 3, 1–55.

SMIDT, E. L. B., 1944: The effects of ice winters on marine littoral faunas. Folia Geogr. Danica, **2,** 3, 1–16.

SMIDT, E. L. B., 1951: Animal production in the Danish Waddensea. Medd. Komm. Danm. Fisk.-og Havunders., Serie Fisk., **11,** 1–51.

STEPHEN, A. C., 1930: Studies on the Scottish marine fauna. Additional observations on the fauna of the sandy and muddy areas of the tidal zone. Trans. Roy. Soc. Edinb., **56,** 521–535.

STEPHEN, A. C., 1931: Notes on the biology of certain Lamellibranchs on the Scottish coast. Journ. Mar. Biol. Ass., **17,** 277–300.

STEPHEN, A. C., 1932: Notes on the biology of some Lamellibranchs in the Clyde area. Journ. Mar. Biol. Ass., **18,** 51–68.

TAMMES, P. M. L. and A. D. G. DRAL, 1955: Observations on the straining of suspensions by mussels. Arch. Néerl. de Zool., **11,** 87–112.

THAMDRUP, H. M., 1935: Beiträge zur Ökologie der Wattenfauna auf experimenteller Grundlage. Medd. Komm. Danm. Fisk.- og Havunders. Serie Fisk., **10,** 2, 1–125.

THORSON, G., 1950: Reproductive and larval ecology of marine bottom invertebrates. Biol. Rev., **25,** 1–45.

THORSON, G., 1951: Zur jetzigen Lage der marinen Bodentier-Ökologie. Verh. Deutsch. Zool. Geselsch. Wilhelmshaven 1951, 276–327.

VEEN, J. VAN, 1936: Onderzoekingen in de Hoofden in verband met de gesteldheid der Nederlandsche kust. Den Haag. Thesis.

VERWEY, J., 1952: On the ecology of distribution of cockle and mussel in the Dutch Waddensea, their rôle in sedimentation and the source of their food supply. Arch. Néerl. de Zool., **10,** 171–239.

VERWEY, J., 1955: Annual report of the Zoological Station of the Netherlands Zoological Society for the year 1953. Arch. Néerl. de Zool., **11,** 143–153.

WALTON, C. L., 1920: Notes on the shell of *Cardium edule*. Trans. Liverpool Biol. Soc., **34,** 143–146.

WILLEMSEN, J., 1952: Quantities of water pumped by mussels (*Mytilus edulis*) and cockles (*Cardium edule*). Arch. Néerl. de Zool., **10,** 153–160.

WILSON, D. P., 1952: The influence of the nature of the substratum on the metamorphosis of the larvae of marine animals, especially the larvae of *Ophelia bicornis* Savigny. Ann. de l'Inst. Océanogr., **27,** 49–156.

WINCKWORTH, R., 1921: Giant race of *Cardium edule* L. Journ. of Conchol., **16,** 157.

WOHLENBERG, E., 1937: Die Watten-Lebensgemeinschaften im Königshafen von Sylt. Helg. Wiss. Meeresunters., **1,** 1–192.

THE POSTEMBRYONIC DEVELOPMENT OF THE PRONEPHROS IN CLUPEA HARENGUS L.

by

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(Amersfoort)

In our publication of 1954 we preferred BURLEND's derivation of the kidney of vertebrates to the hypothesis of BALFOUR and SEDGWICK regarding the fundamental structure of this organ. For many years, however, the hypothesis of BALFOUR and SEDGWICK has been fairly generally accepted. It was therefore necessary the enlarge the factual basis of our conclusions of 1954. In this paper some more evidence is offered. Our choice fell on the herring, because it is fairly akin to the milkfish, *Chanos chanos* (Fork.).

Fig. 1 shows a graphical reconstruction of the anterior part of the kidney of a larva of 10 mm[1]. The kidney is formed by two nephrons; the glomeruli and the Bowman's capsules have fused, the tubules run almost straight in caudal direction, a condition we also found in some other primitive Teleostei (1938). The initial sections of the tubules, as far as A, are lined with the same sort of cells, out of which the Bowman's capsule is made up. The rest of the tubules, as far as indicated in the drawing, is formed by a cuboidal epithelium with brush-border. The glomerulus lies in a shunt of the aorta, the efferent vessel meeting the aorta again near the root of the art. mes. ant. Its position is indicated with regard to the ventral roots of the spinal nerves, as rudiments of the vertebrae are still lacking. In numbering these ventral roots we have taken into account that in Teleostei they are preceded by three pairs of spino-occipital nerves.

[1] The dimensions refer to material fixed in Bouin's fluid, contraction ± 20 %.

In larvae of about 20 mm, fig. 2, the efferent vessel of the glomerulus no longer joins the aorta, its opening having shifted a little way along the initial part of the art. mes.ant. This way of bloodsupply is also found in older larvae and as long as this condition continues the glomerulus remains conspicuous for its size. Compared with the previous stage it has shifted in caudal direction. The anterior wall of the Bowman's capsule shows a depression. We should not have paid any attention to this groove, attributing it to fixation, if it did not occur

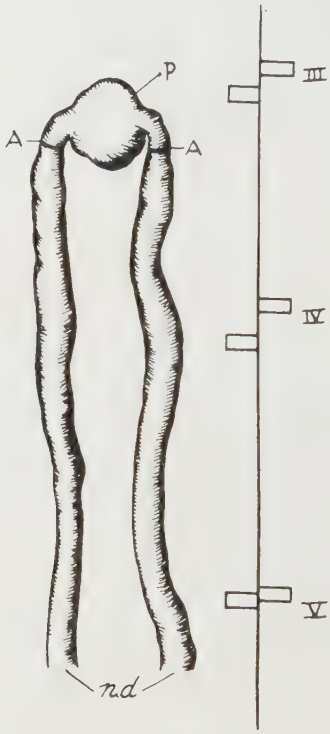


Fig. 1. Herring larva 10 mm, reconstruction of anterior part of kidney, × 185, dorsal view; P. pronephric glomerulus, n.d. archinephric duct, II–V indication of ventral roots of spinal nerves.

similarly in the following stage. Apparently the glomerulus has been dragged along by the bloodvessels shifting backwards. The Bowman's capsule has not been able to keep up with this movement, being anchored as it is to the nephric ducts. The wall of the Bowman's capsule generally consists of flagellate, cuboidal cells with nuclei situated apically. Where the capsule passes into the archinephric duct,

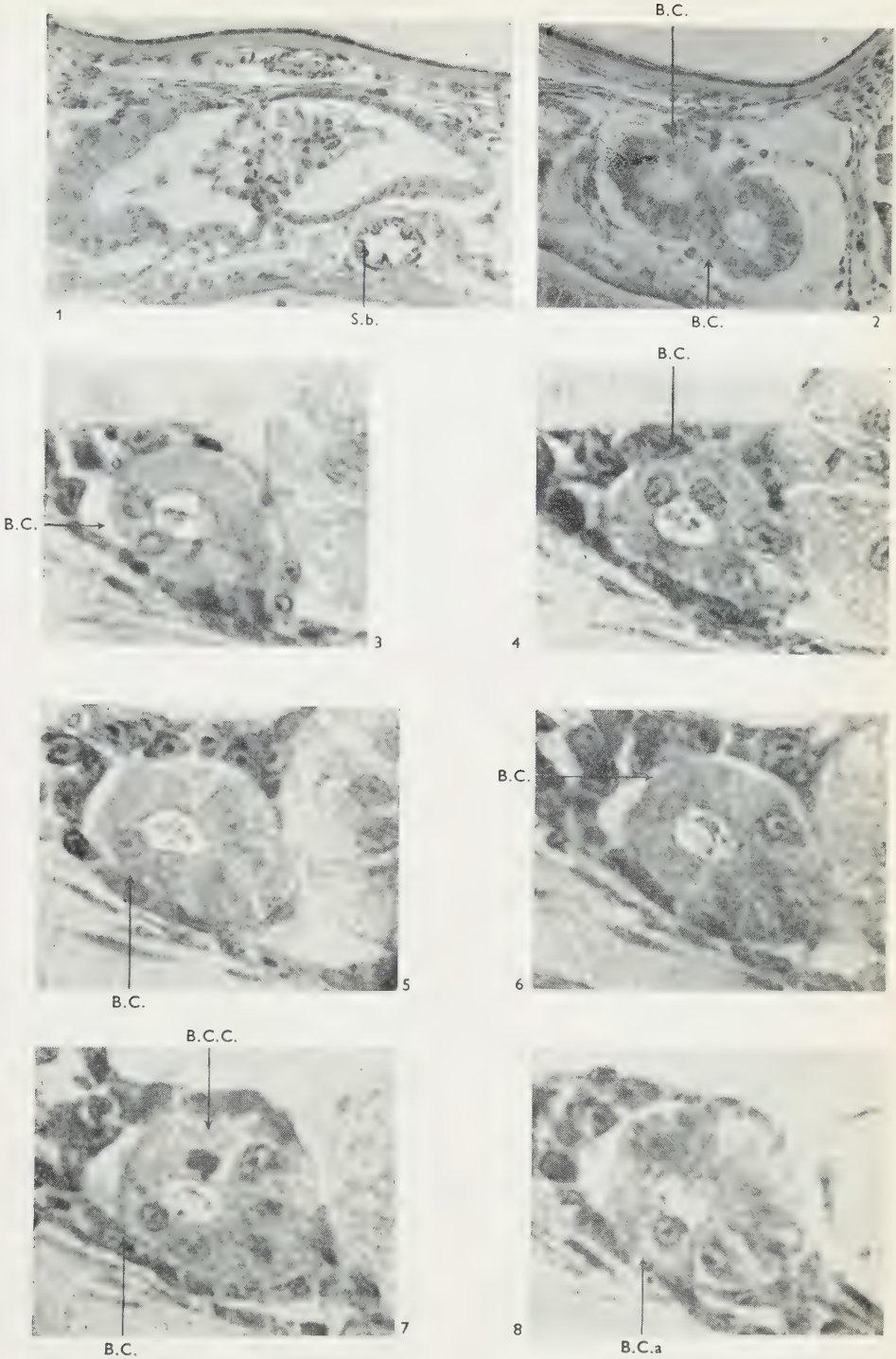
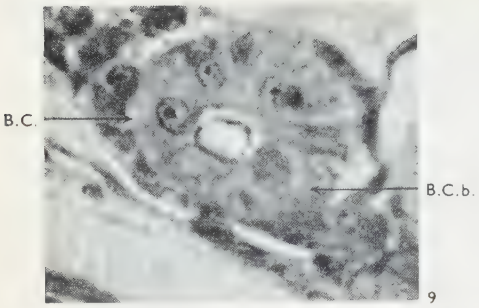
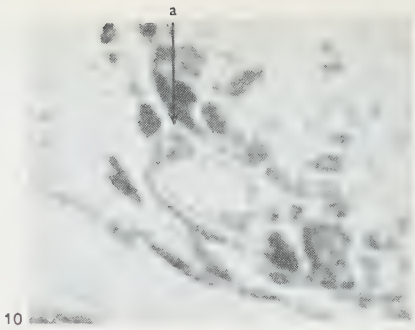
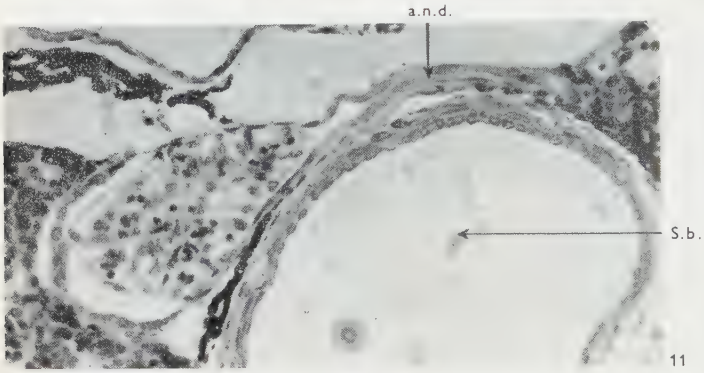
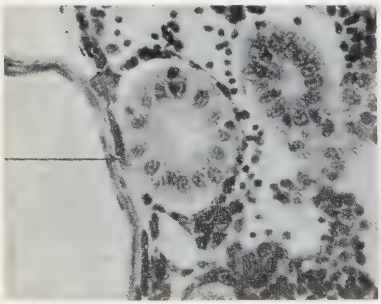


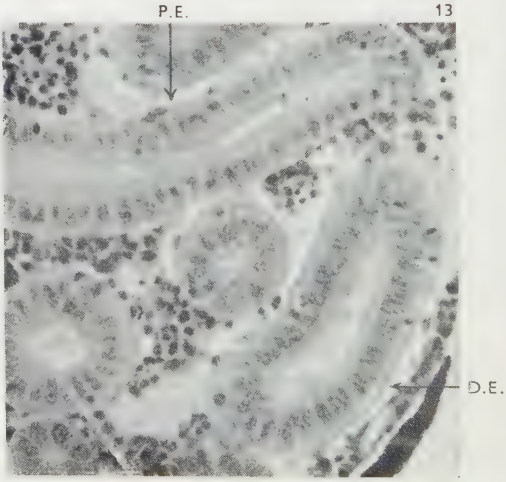
Foto 1. Section through 1 of fig. 2, 45 × 10, 7 μ, haematoxylin + eosine. To the right cuboidal epithelium, to the left pairs of cells among the columnar epithelial cells, S.b. swimming-bladder. – 2. Section through 2 of fig. 2, 45 × 10, 7 μ, haem. + eosine, B.C. Bowman's capsule cells. – 3–9. Seven successive sections through part 3 of fig. 3, 1/12 oil × 10, 7 μ, haem. + eosine. B.C., nuclei of the Bowman's capsule-cells; B.C.C. division of a Bowman's capsule-cell; B.C.a., B.C.b. Bowman's capsule cell among young cells.











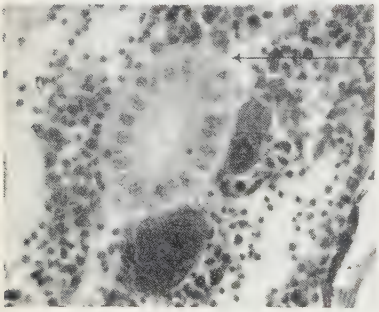


Foto 10. Section through 4 of fig. 4, 1/12 oil × 10, 7 μ, haem. + eosine; a. typical nucleus a Bowman's capsule-cell. – 11. Young herring, 28 mm, cross section of pronephric glomerulus, 45 × 10, 7 μ, haem. + eosine, a.n.d. right arch. duct, S.b. swimming-bladder. – 12. Section through 5 of fig. 5, 45 × 10, 7 μ, haem. + eosine, P.E. proximal epithelium. – 13. Section through part of secondary nephron of a young herring of 28 mm, 45 × 10, 7 μ, haem. + eosine, P.E. proximal, D.E. distal epithelium. – 14. Section through 6 of fig. 6, 45 × 10, 7 μ, haem. + eosine, P.E. proximal epithelium, black spots: cross section of rudiment of sec. nephron.

these cuboidal cells have been replaced by columnar ones, still provided with flagels and among these we see pairs of cells lying one above the other (photo 1). The columnar cells represent apparently the incipient, the pairs of cells the closing phase in the division of the

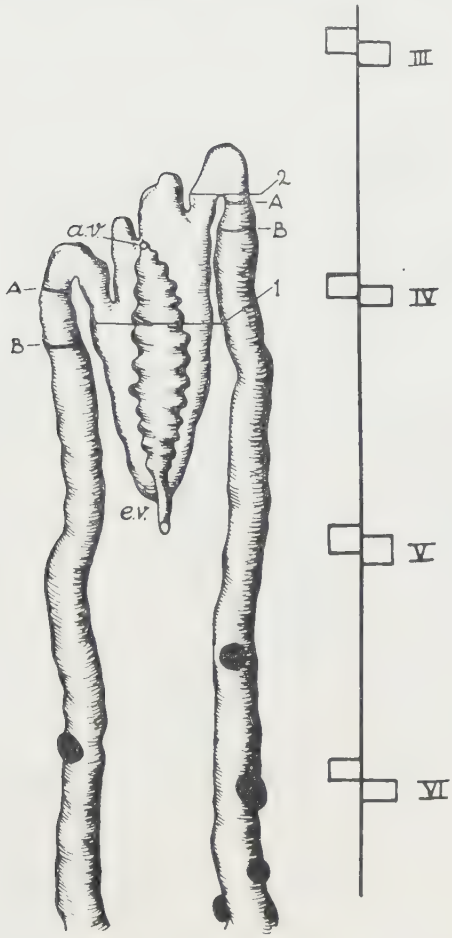


Fig. 2. Herring larva 20 mm, reconstruction of anterior part of kidney, × 107, dorsal view; a.v. afferent vessel-, e.v. efferent vessel of glomerulus, III–VI indication of ventral roots of spinal nerves, black spots indicate the rudiments of secondary nephrons.

cells of this part of capsule wall. The new material has been used for the lengthening of the nephric ducts, which has become necessary, because of the shifting of the capsule. As far as A, photo 2, we find, among the

other cells of the tubules, cells which are typical for the Bowman's capsule. When stretched, the Bowman's capsule behaves here as a viscous membrane, the elements of which move along one another. Distally to A, the epithelium just discussed passes into a columnar epithelium, possessing cells with brush-border, a layer of clear proto-

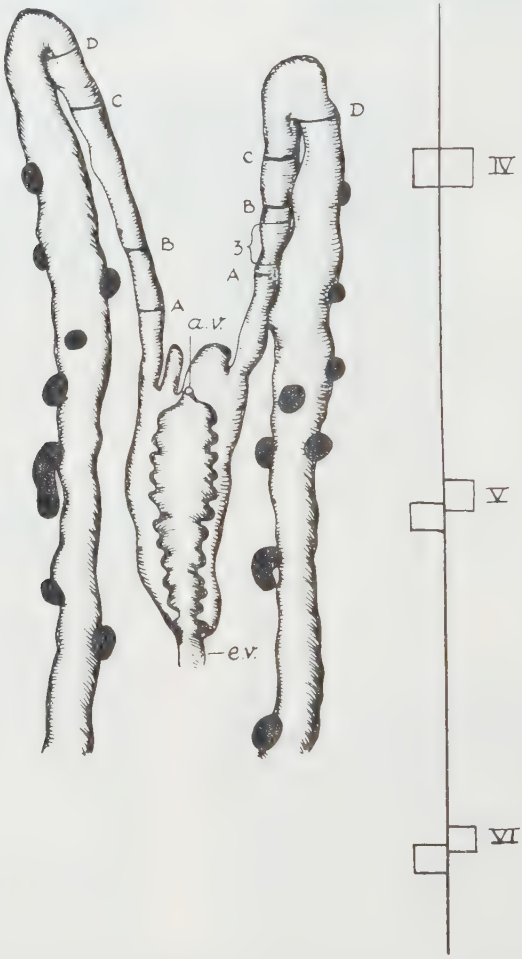


Fig. 3. Herring larva 29 mm, reconstruction of anterior part of kidney, × 107, dorsal view. See Fig. 2 for further explanation.

plasm under the basal granules and central nuclei. We indicate it as distal epithelium (Holstvoogd 1954). The initial part (A–B) has still basophile protoplasm.

Fig. 3 refers to a larva of 29 mm. Compared with the previous stage

the glomerules has shifted again in caudal direction, and in connection with this shifting, the medial limbs of the nephric ducts have also lengthened again. As far as A the latter are formed by capsule-epithelium. Distally to A they possess, besides capsule-cells, young cells which are taller than the first mentioned, and have a central nucleus and basophile protoplasm, photo 3 9. In distal direction they become more and more numerous and beyond B they exclusively form the wall of the nephric ducts. From the way in which the capsule-cells are spread out over the segment A B, we must conclude that this segment has arisen from material originating from the wall of the Bowman's capsule. From what material the segment B C originates is hard to tell with certainty. Beyond C the epithelium of the nephric ducts assumes the character of distal epithelium; the initial part, C D, being still basophile. Beyond D we find fully differentiated distal epithelium.

In larvae of 35 mm the glomerulus lies more or less in its definite position, at least in cranio-caudal direction, fig. 4. To the right the Bowman's capsule shows a depression (x) caused by the growth of the swimming-bladder. The initial section of each archinephric duct consists of the same epithelium as the Bowman's capsule. This is followed by the segment A B, which is formed by a "pavement"-epithelium with basophile protoplasm and round nuclei, bulging inwards and outwards. We consider this as capsule-epithelium which has been flattened by stretching, photo 10. Coming to B, we see the beginning of a regeneration of the epithelium. The cells become taller, the nuclei get a central position, but the protoplasm still remains basophile. After some sections we can also distinguish a brush-border. Whether we have to do here with young distal or young proximal epithelium, can as yet not be determined. To the left, where the regeneration starts earlier, we find as far as B_1 here and there still clusters of Bowman's capsule-cells. The segment C D is formed by distal epithelium, which passes at D into the epithelium of the collecting ducts. The latter is distinguished from the distal epithelium by its lack of the layer of clear protoplasm under the basal granules and its lower brush-border.

Fig. 5 shows a reconstruction of the anterior part of the kidney of a herring of 28 mm in length, which has already passed through its metamorphosis; of the younger nephrons only the openings are indicated. The pronephric glomerulus has been pushed to the left by the growing swimming-bladder, while the afferent vessel has become disconnected from the aorta and has disappeared. For the time being, the original efferent vessel sees to the bloodsupply. The blood from the glomerulus now flows away into the lymphoid tissue just as in an ordinary Malpighian body. In photo 11 we see how the righthand pronephric duct finds with difficulty its way across the swimming-

bladder. The Bowman's capsule-epithelium, which in the previous stage we still found in the initial part of the nephric ducts, has now also

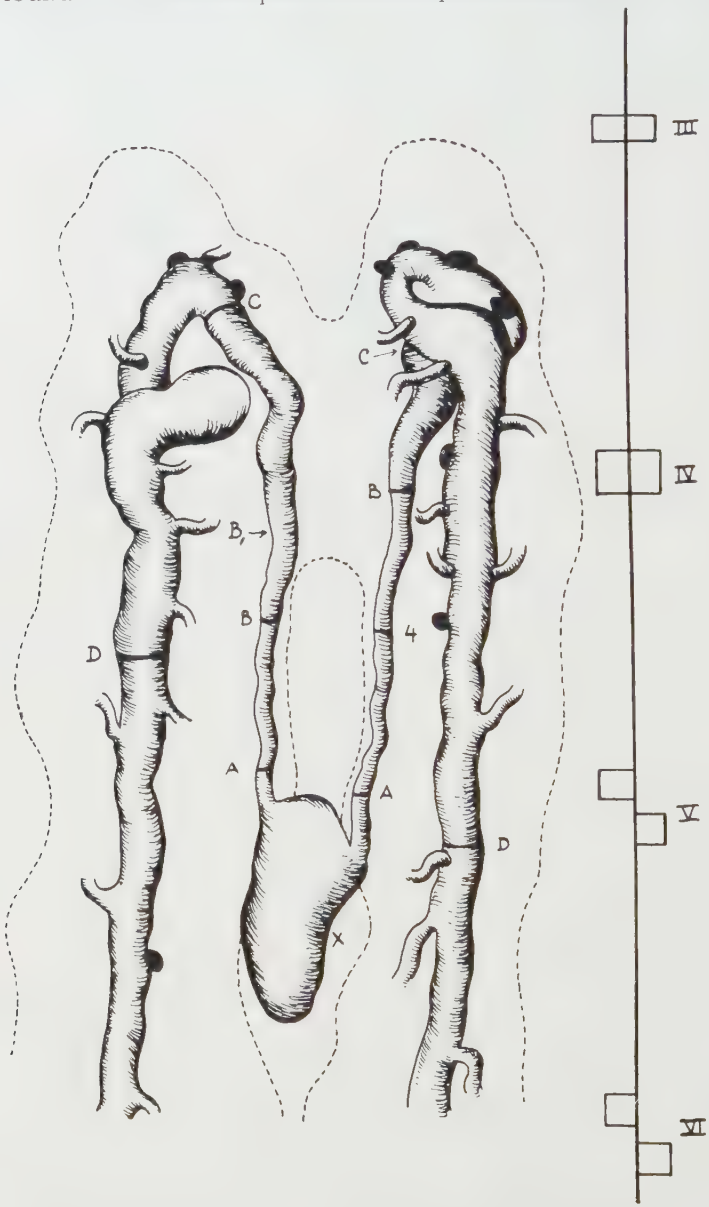


Fig. 4. Herring larva 35 mm, reconstruction of anterior part of kidney, × 107, dorsal view; of the secondary nephrons only the openings are indicated. The dotted lines indicate the boundary of the kidney. See for further explanation fig. 2.

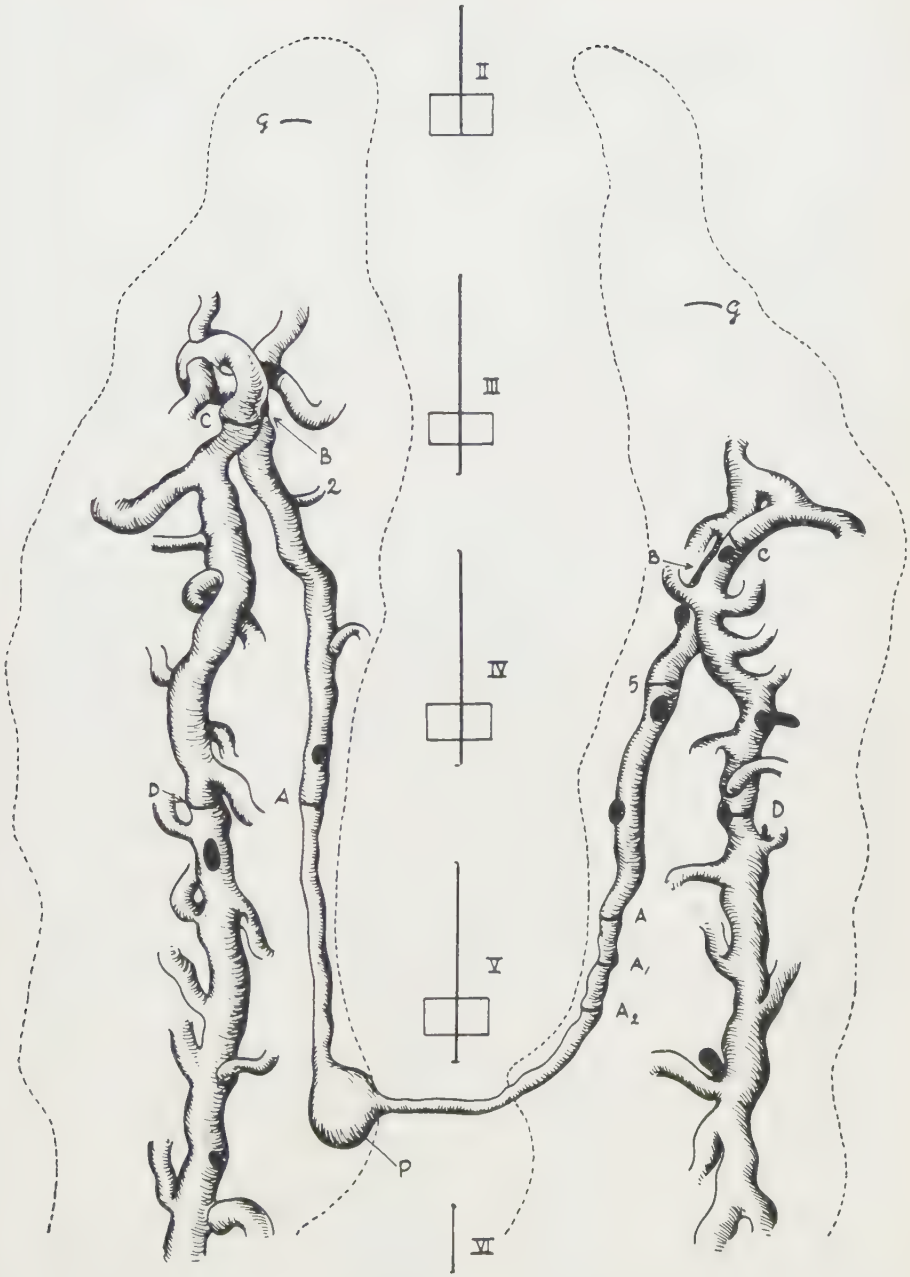


Fig. 5. Young herring 28 mm, reconstruction of anterior part of kidney, ⌃ 107, dorsal view; II–VI the lines indicate the ventral rudiments of vertebrae, the rectangles the ventral roots of spinal nerves. The dotted lines indicate the boundary of the kidney, cranial to G lymphoid tissue only. Of the secondary nephrons only the openings are indicated. Black spots indicate the rudiments of nephrons, P. pronephric glomerulus.

changed into flattened epithelium, possessing cells with basophile protoplasm and nuclei which often bulge slightly. The section A–B is formed by an epithelium, consisting of cells with clear to finely striped protoplasm, a high brush-border, and nuclei situated in the basal half of the cells. We indicate it as proximal epithelium, photos 12, 13. To the left the flattened epithelium passes straight into the proximal epithelium, but to the right the section A_2–A_1, consisting already of proximal epithelium, is followed by a section A_1–A, formed by flattened epithelial cells. The epithelium of section B-C is in a state of regeneration. In some cells the nuclei occupy a basal position (old proximal cells), but they alternate irregularly with cells with central nuclei (new distal cells). This irregular epithelium passes into the distal epithelium of section C-D, while beyond D this distal epithelium passes again into the epithelium of the collecting ducts.

Fig. 6 refers to a young herring of 35 mm. Of this animal we have reconstructed only the left archinephric duct; the one on the right has meanwhile lost its connection with the glomerulus and could therefore not be traced from its start. Compared with the previous stage, the various kinds of epithelium have moved along in proximal direction with the exception, however, that the initial part of the archinephric duct, as far as A, still consists of flattened epithelial cells with basophile protoplasm, but they are slightly taller than in the previous stage. This initial segment passes immediately into proximal epithelium, photo 14. It runs as far as B, but from B_1 onwards it is becoming more and more irregular.

In a slightly older animal, fig. 7, the Bowman's capsule passes through a short neck, corresponding in length with the neck-segment of the other nephrons, into the archinephric duct. Apart from this neck segment, the initial part of the archinephric duct, which in the previous stage still possessed flattened epithelial cells, is now formed by proximal epithelium, and on this part secondary nephrons have already arisen; it runs as far as A.

In young herrings of 50 mm we find instead of the pronephric glomerulus a cavity bordered by connective tissue.

SUMMARY AND CONCLUSIONS

In our investigation the easily recognizable Bowman's capsule-cells have played a part comparable to those of the vitally stained spots used in experimental embryology, and so it could be determined that the initial parts of the archinephric ducts arise by means of the stretching of the Bowman's capsule, supported by cell division. These segments must

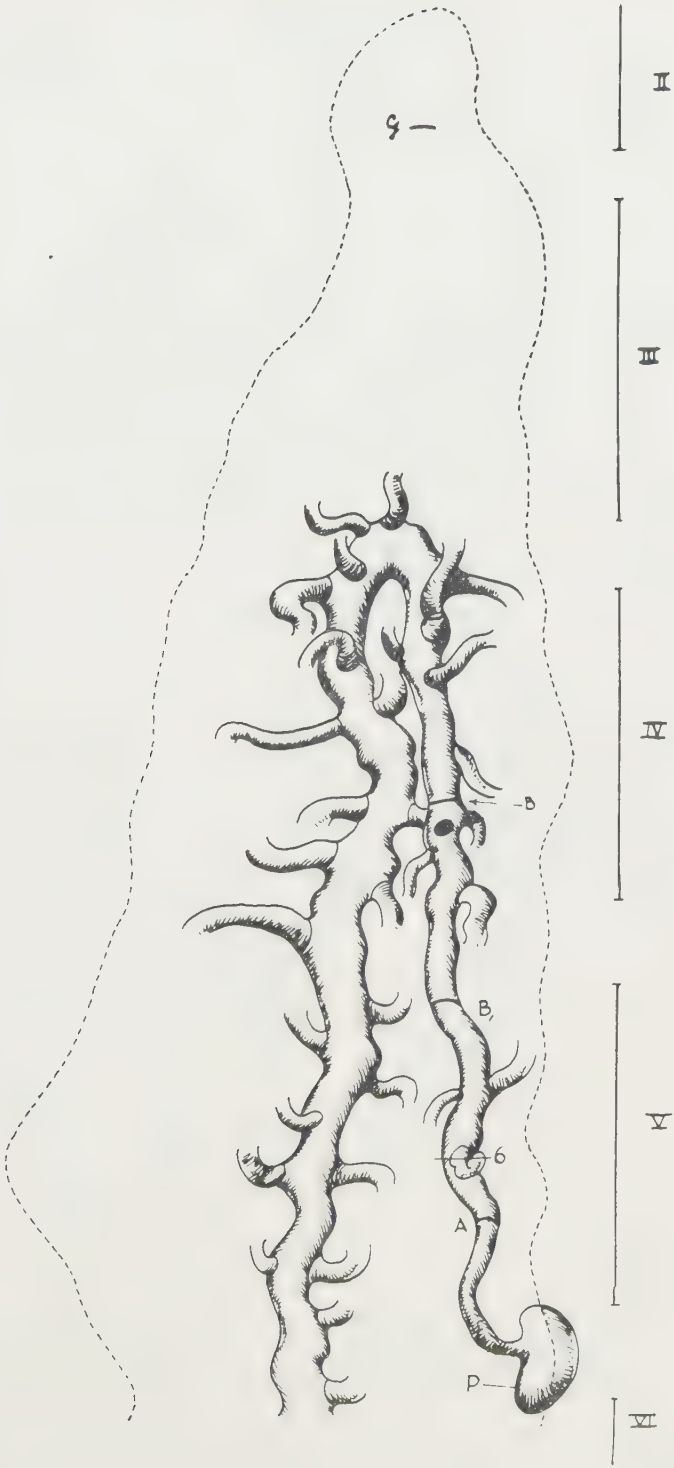


Fig. 6. Young herring 35 mm, reconstruction of anterior part of kidney, dorsal view, × 122. See for further explanation fig. 5.

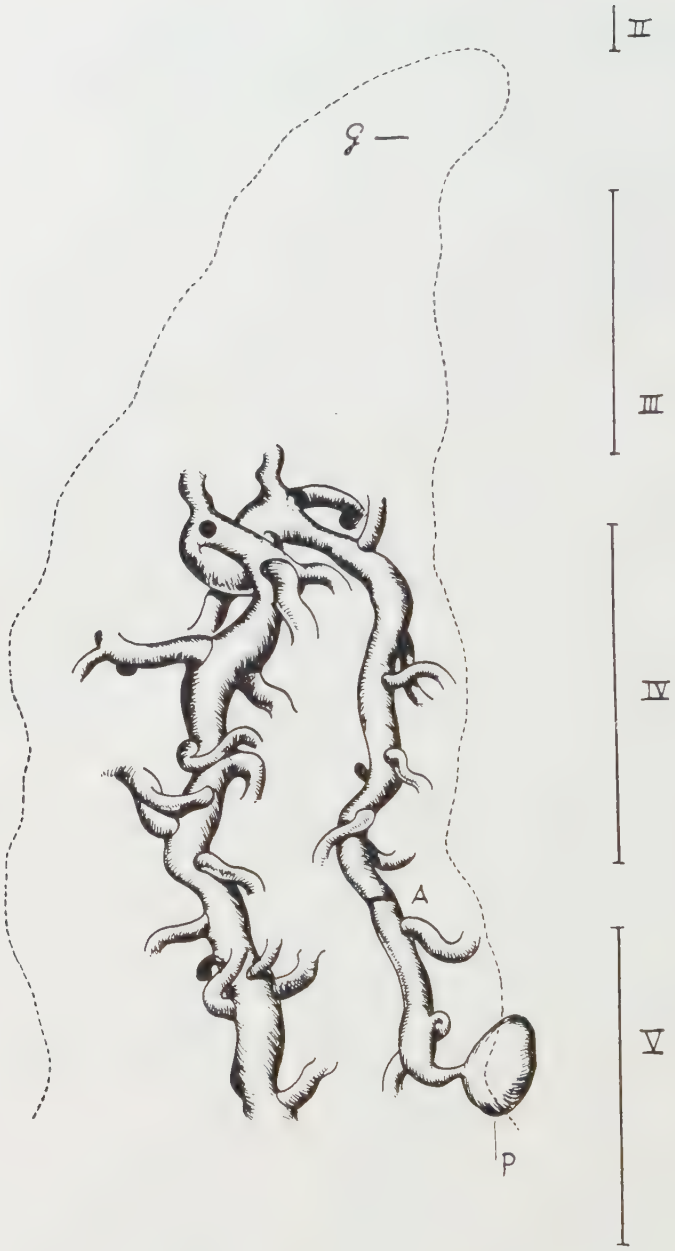


Fig. 7. Young herring 35 mm, reconstruction of anterior part of kidney, dorsal view, × 122. See for further explanation fig. 6.

therefore be considered as parts of the pronephros. They represent pronephric tubules which lagged behind in development. On these initial segments secondary tubules originate, which in their turn have to be considered as secondary pronephric tubules. However, the occurrence of seconday pronephric tubules is inconsistent with the hypothesis of BALFOUR and SEDGWICK, (FELIX, 1904–'06).

To speak here of pronephric tubules and secondary pronephric tubules is virtually a reasoning ad absurdum. Pronephric tubules belong to a much earlier stage in the development than are discussed in this paper. On the other hand, these tubules originate from the glomerulus-capsule and therefore belong to the pronephros. Here the hypothesis of BALFOUR and SEDGWICK proves to be untenable.

Let us just pay some more attention to the kidney of the larva of 10 mm. It is composed of two tubules, each with a dilated anterior extremity, in which there is a glomerulus. Over their total length secondary nephrons may develop on these tubules. We have to do here with an organ fairly akin to that which BURLEND (1931) postulated when he derived the kidneys of vertebrates from a pair of folds in the somatopleura. These folds closed into two ducts, which remained, however, in communication with isolated parts of the coelom by means of a number of openings; the latter developing into renal tubules, the former into glomeruli. The development of the kidney of Mixinoidea, Teleostei, Dipnoi, Urodela and Anura fits well in with BURLEND's hypothesis. The segmental development of the renal units in Selachii and Gymnophiona finds a ready explanation in the fact that a slightly larger part of the mesoderm is segmented in these animals.

A permanent pronephros is according to the older hypotheses an absurdity, hence the scepticism with which the first papers on this subject were received (HOLSTVOOGD 1954). For BURLEND's hypothesis it constitutes no difficulties. One does not, however, put the question correctly, when one asks if an animal has a permanent pronephros; one should ask, which part of the primary nephrons persists in the mature animal and, in doing so, one should not lose sight of the fact that to these primary nephrons also belong the archinephric ducts. In *Chanos* the entire primary nephrons persist. The same holds good for *Clupea*, except for the Malpighian body, to which the pro nephros glomerulus and its capsule has been reduced after the loss of its original vas afferens. The disappearance of this Malphighian body is to a certain extent to be compared to the falling off of the terminal bud of the plant after which a lateral one takes over its function.

The herring larvae we received from the Zoological station at Den Helder; the other material from the Department of Comparative Physiology at Utrecht. The microscopic slides were prepared in the Department of Prop. Zoology of the University of Amsterdam.

REFERENCES

BURLEND, T. H., 1931: The origin of the archinephric duct of Vertebrates. Amer. J. Anat., xx.

FELIX, W. und BÜHLER, 1904–1906: Die Entwicklung der Harn- und Geschlechtsorgane. O. Hertwig, Handbuch der Entw. lehre der Wirbeltiere. Bd. 3, Tl. 1.

HOLSTVOOGD, C., 1938: De bouw en ontwikkeling der Beenvissennier. Handelingen v. h. achtste Ned.-Indische Natuurwetenschappelijk congres, Soerabaja, 381–385.

HOLSTVOOGD, C., 1954: The postlarval development of the pronephros in *Chanos chanos* (Forsk.), Arch. Néerl. de Zoologie, xx, 386–404.

ON THE PRODUCTION OF HORMONES IN THE THYROID GLAND OF BIRDS (COCKERELS)

A QUANTITATIVE STUDY WITH THE HELP OF RADIOIODINE AND ANTITHYROID DRUGS

by

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I. INTRODUCTION

During the last 15 years the study of thyroid physiology has progressed greatly. This progress was due to the development of new physical and chemical techniques, for instance the application of radioiodine (mostly in thyroid studies I^{131} is used), of antithyroid drugs, and of paperchromatographical and radioautographical techniques. With the aid of these expedients our knowledge about the biosynthesis of hormones in the thyroid gland advanced greatly.

In surveying the literature on the thyroid there is a striking preponderance of investigations with mammals as experimental animals, whereas only incidentally attention has been drawn to other classes of the vertebrates.

Next it is clear that there have been only few investigations giving a quantitative account of the processes inside the thyroid gland. Studies are mostly either qualitative, or they inform about the quantitative release of hormones by the thyroid gland, without going into the synthesis of hormones within the thyroid gland.

We have tried to investigate the biosynthesis of thyroid hormones in *birds*, using cockerels as experimental animals. We aimed to study *quantitatively* the processes which govern the production of thyroid hormones. Moreover we tried to compare the results of our investigations with the experimental data collected about the sequence of events in the production of thyroid hormones in other classes of vertebrates, especially in mammals.

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II. THE PRESENT KNOWLEDGE ABOUT THYROID PROCESSES AND IODINATED AMINO ACIDS

We will first give an introduction to the main lines of thought on the biosynthesis of thyroid hormones. As the investigations are performed mainly with mammals as experimental animals, we will begin by surveying the literature on this group of animals. Part of the papers are referred to in the discussion of our own experiments.

Much of the literature in this field has been adequately reviewed by LEBLOND (1951), ALBERT (1952), GROSS and PITT-RIVERS (1952), ROCHE and MICHEL (1951, 1954, 1955, 1956), and BARKER (1955).

First, one of the principal properties of thyroid tissue is the ability to trap iodide. The thyroid can maintain an iodide concentration higher than that of the circulating blood, in rats the ratio of thyroid to blood iodide averaging 25 in the normal animal and 280 in animals with hyperplastic glands (VANDERLAAN and VANDERLAAN, 1947).

There is some divergence of opinion about the site of this iodide-trapping mechanism inside the thyroid follicle. At first, for instance, LEBLOND and GROSS (1948) and GROSS and LEBLOND (1951) assumed that the iodide was concentrated within the follicle-cells, whereas the organic iodine was present in the colloid. The binding of the iodide in the thyroglobulin (and the conversion of iodide to elementary iodine) was assumed to happen in the apex of the epithelium cells. Afterwards PITT-RIVERS and TROTTER (1953) and DONIACH and LOGOTHETOPOULOS (1955) demonstrated the presence of the concentrated iodide in the colloid in rats, pretreated with propylthiouracil or thiouracil (which inhibits the organic binding of iodine) before they were injected with radioiodide. By autoradiography of the thyroid glands they were not able to demonstrate the radioiodide in the follicle-cells. NANDI, PODDAR and PYNE (1956), however, showed, 15 min. after an injection with radioiodide in rats, twice as much iodide in the cells as in the colloid, 1 hour after the injection the ratio iodide in the cells to iodide in the colloid being 1 : 3. This ratio increased afterwards. In this study the animals were not pretreated with antithyroid drugs. The authors claim that their results seem to suggest that in the case of rats, kept on a normal diet, iodine is first taken up by cells, where it is incorporated into the protein, and this iodinated protein then diffuses into the colloid (cf. LEBLOND and GROSS 1948). HALMI, STUELKE and SCHNELL (1956) however, studying the so called 'iodide space' in rats, treated with $KClO_4$, concluded that in the trapping the whole cell is not involved, but the iodide pump is located in the apex of the cells. From this apex the iodide, which has entered the cell by diffusion, is stored in the colloid.

Afterwards this iodide is oxidized to elementary iodine, which is then bound, presumably by enzymic activity, to amino acids of the thyroid protein. So the iodinated thyroglobulin is formed inside the lumina of the thyroid follicles (LEBLOND and SUE 1941, LEBLOND and GROSS 1948, GROSS and PITT-RIVERS 1952, DONIACH, HOWARD and PELC 1953, NADLER, LEBLOND and BOGOROCH 1954, WOLLMAN and WODINSKY 1955).

Then the thyroid hormones are synthesized inside the thyroglobulin.

At first especially the formation of thyroxine (Tx) by condensation of two molecules diiodotyrosine (DIT), as suggested by HARINGTON and BARGER (1927), has drawn attention (PITT-RIVERS 1948, 1952). Afterwards the presence of other iodinated amino acids, besides Tx and DIT, has been shown. Monoiodotyrosine (MIT) first was demonstrated by FINK and FINK (1948); monoiodohistidine (MIH) by ROCHE, LISSITZKY and MICHEL (1951); 3 : 5-diiodothyronine (3 : 5-DITh) by ROCHE, LISSITZKY and MICHEL (1951); 3 : 5 : 3′-triiodothyronine (3 : 5 : 3′-TITh) at the same time by GROSS and PITT-RIVERS (1952), and ROCHE, LISSITZKY and MICHEL (1952); 3 : 3′ : 5′-triiodothyronine (3 : 3′ : 5′-TITh) and 3 : 3′-diiodothyronine (3 : 3′-DITh) by ROCHE, MICHEL, NUNEZ and WOLF (1955).

Next the thyroglobulin is broken down by proteolytic enzymes (DE ROBERTIS 1941, 1954; MCQUILLAN and TRIKOJUS 1953, 1954). By this proteolysis the iodinated amino acids are set free, as demonstrated by GROSS, LEBLOND, FRANKLIN and QUASTEL (1950) in rats. MIT, DIT and probably also MIH are deiodinated (ROCHE, MICHEL, MICHEL and LISSITZKY, 1953; ROCHE, MICHEL, MICHEL, GORBMAN and LISSITZKY, 1953), after which the iodide can be used again in the synthesis of thyroglobulin.

On the other hand the iodothyronines, probably by mere diffusion, as suggested by GROSS and LEBLOND (1951) for Tx in rats, are released into the blood.

Up to the present in the blood have been demonstrated:

Tx (LAIDLAW 1947; TAUROG and CHAIKOFF 1947), 3 : 5 : 3′-TITh (GROSS and PITT-RIVERS 1952; ROCHE and MICHEL (1952), 3 : 3′ : 5′-TITh (ROCHE, MICHEL, NUNEZ and WOLF 1955), and 3 : 3′-DITh (ROCHE, MICHEL, NUNEZ and WOLF 1955; ROCHE, MICHEL, WOLF and NUNEZ 1956).

The physiological activity as thyroid hormones has been shown for the abovementioned amino acids.

Now we will review the literature on iodine metabolism in the thyroid gland in the other classes of vertebrates, especially in birds, after which we will refer to some data on the iodine metabolism in invertebrates.

As was mentioned in the introduction, only a few papers has been published on the physiology of the thyroid gland in birds. A survey is given by FLEISCHMANN (1951). He discusses among other things the physiological activity of Tx in birds, the results of thyroidectomy, and the daily requirement of Tx, determined with the aid of the goitre prevention test (♂ chickens require 2–3 γ Tx daily during the first weeks of life). First WOLFF and CHAIKOFF (1947) investigated the presence of different amino acids in birds. They concluded (1947) that in verte-

brates 25–32% of the iodine in the thyroid gland is present in Tx: the amount in chickens (25%) was the lowest quantity in the animals studied (they investigated fishes, amphibians, birds and mammals).

In a subsequent paper TAUROG, TONG and CHAIKOFF (1950) determined in birds and rats the amount of iodine in inorganic iodide, MIT, DIT and Tx. In birds they observed 14% inorganic iodine, 22% MIT, 24% DIT and 22% Tx, the remainder not being identified. They concluded that probably in birds, as compared with rats, there is more MIT and less DIT, whereas the relative amounts of Tx in both are of the same order.

The results of these investigations however are not wholly reliable, because the authors hydrolysed their material with NaOH. In this manner, as STANLEY (1953) has shown, the quantity of Tx may be too high. This is admitted by TAUROG, POTTER and CHAIKOFF (1955), who say that "It is becoming increasingly clear that strongly alkaline hydrolysis may introduce serious errors into the analysis of mixtures of I^{131} compounds" (p. 124).

As far as we know this is the one paper which goes into the thyroid gland of birds in the way we have tried to do it.

In addition to these investigations there are some more papers on the start of thyroid function during the embryogenesis of birds. First STOLL and BLANQUET (1952) have shown that trapping of iodide in the thyroid gland of chicken embryos starts at the 7th day of incubation. This paper (affirmed by MITSKEVICH and MAMUL 1953) does not reveal anything about the form in which the iodine is present in the gland. WOLLMAN and ZWILLING (1953) ascertained that the organic binding of $iodine^{131}$ probably does not happen in thyroid glands of chickens up to the 7th day of incubation, though it happens at the 9th day of incubation.

In a paperchromatographical study BLANQUET, STOLL, MARAUD and CAPOT (1953) showed the presence of iodide, DIT and Tx at the 12th day of incubation but they failed to demonstrate MIT. They suppose that MIT only exists as an intermediate ("son existence pouvant être transitoire"). In connection with other papers this explanation seems to us to be rather unacceptable.

On the 8th day of incubation CARPENTER, BEATTIE and CHAMBERS (1954) found a very small binding of iodine in thyroids of chickens. This binding starts simultaneously with the appearance of small P.A.S.-positive droplets, which is supposed to indicate the beginning of the production of colloid. At the 16th day of incubation the amount of concentrated radioiodine had increased greatly. This applies also to glands, cultivated in vitro from the 8th day of incubation. When studied by autoradiography the presence of ring-structures, situated above the bor-

derline of the colloid and the apex of the epithelium cells, was noteworthy.

Finally in an extensive study TRUNNELL and WADE (1955) concluded: at the 5th day of incubation the amount of radioiodide in the thyroid gland is 50 times as high as the amount in the cardiac muscle. All of the activity in these thyroids was removable by treatment with the trichloroacetic alcohol solution, indicating that little or no binding and probably no conversion to iodine had taken place. This situation existed as late as 7½ days, and almost for another 24 hours. At 8½ days however MIT was first synthesized, roughly ¾ day later followed by DIT. About ½ day later the first, faint smudge of Tx was seen. It was not until 10 days that Tx was produced in easily detectable quantities.

They reported in this paper that they could not observe any 3 : 5 : 3′-TITh, and only very small quantities of MIH. The impossibility to detect 3 : 5 : 3′-TITh may be caused by their chromatographical procedure, in which Tx and 3 : 5 : 3′-TITh were not clearly separated.

Resuming, we may conclude that in birds MIT, DIT, MIH and Tx have been demonstrated. The proportions of the amino acids are different from those in mammals (TAUROG, TONG and CHAIKOFF 1950), but it is impossible to specify these differences, in consequence of the deficiencies in the techniques used.

In reptilians WOLF and CHAIKOFF (1947) demonstrated the presence of Tx.

SHELLABARGER, GORBMAN, SCHATZLEIN and MCGILL (1956) studying the thyroid function in tortoises and turtles, demonstrated the presence of MIT, DIT, 3 : 5 : 3′-TITh and Tx in the tortoise *Terrapene carolina* and in the turtle *Pseudemys floridanus*. They failed to observe any relation between the accumulation of the radioiodine and the capacity to synthesize thyroid hormones. In amphibians BRADLEY (1951) observed a decrease in the uptake of radioiodine in the thyroid gland under the influence of different antithyroid drugs. He did not analyse the presence of iodinated amino acids.

In fishes Tx was detected by WOLF and CHAIKOFF (1947) in *Scyllium* spec.

GORBMAN, LISSITZKY, MICHEL and ROCHE (1952) in *Scylliorhinus (Scyllium) canicula* showed the presence of MIT, DIT and Tx. The same applies to investigations of BERG and GORBMAN (1953) in *Xiphophorus maculatus*, BERG and GORBMAN (1954) in *Carassius auratus*, FONTAINE, LELOUP and OLIVEREAU (1953) in *Anguilla anguilla*, and GORBMAN and BERG (1955) in *Fundulus heteroclitus*, *F. majalis* and *F. diaphanus*.

Finally LELOUP (1956) succeeded in demonstrating, beside the forementioned iodinated amino acids, 3 : 5 : 3′-TITh in *Periophthalmus koelreuteri*.

In lampreys LELOUP and BERG (1954) showed the presence of MIT, DIT and Tx in larvae of *Lampetra planeri* and *Petromyzon marinus*.

In invertebrates, too, the accumulation of iodine and the presence of iodinated amino acids has been shown.

THOMAS (1956) mentions accumulation of radioiodine in the lateral mucous glands of the endostyl in *Amphioxus lanceolatus*, whereas GORBMAN (1941) showed the uptake of radioiodine in stolonic septa of the ascidians *Styela* and *Ciona*. Both studies werd carried out with the help of radioautography, the compounds in which the iodine was present not being analysed.

GORBMAN, CLEMENTS and O'BRIEN (1954) found accumulation in integumentary glands of *Dolichoglossus kowalewski* (Hemichordata), in the shell, the mantle, the epithelium of the foot and in some parts of the gill-epithelium in *Musculium partumeium* (Lamellibranchia) and in the byssus threads of *Mytilus edulis*.

Moreover, they demonstrated radioiodine in the setae, pharyngeal teeth and cuticle of various polychaetes and in the mandibles and carapace of crustaceans. A weak accumulation could be observed in the epidermis of *Hydra fusca*.

In *Musculium* they found the iodine in the form of several amino acids: MIT, DIT and Tx. Probably in *Nereis virens* (Polychaeta) they detected also DITh.

ANTHEUNISSE and LEVER (1956) observed accumulation in the external parts of setae in *Tubifex* spec. (Oligochaeta) and in the newly formed part of the shell, the epithelium of the mouth, pharynx, radula sheeth, esophagus, upper jaw area and in the footepithelium, liver and kidney of young specimens of *Planorbis corneus* (Gastropoda). In this animals MIT, DIT, 3 : 5-DITh and 3 : 5 : 3′-TITh could be detected.

FRETTER (1952) studied the iodine metabolism in *Helix aspersa*, *H. pomatia*, *Arion hortensis* and *Agriolimax agrestis*. Radioiodine was taken up most easily by digestive cells. Some of it entered lime-cells. Further it was only accumulated in the kidney, whence it was excreted. Only in *Arion hortensis* the I^{131} may be centered around scattered particles of the shell, lying in the mantle cavity.

WHEELER (1950) demonstrated radioiodine in several parts of larvae of *Drosophila gibberosa*.

DRECHSEL (1896) found DIT ("iodogorgonic acid") in the skeleton of corals and ROCHE (1952) mentions that in scleroproteins of Porifera and Cnidaria MIT and DIT, in Gorgonacea also Tx have been detected.

It is evident that iodine accumulation and organic binding of iodine is a general phenomenon in the animal kingdom. The thyroid gland in vertebrates however has a unique position among the tissues which

present these characteristics, as here the products of these processes are of high physiological importance. So far physiological activity of iodinated compounds, as released by the thyroid glands in vertebrates, has not been demonstrated with certainty in invertebrates.

III. TECHNIQUES

The experiments were carried out with 7–10 weeks old cockerels (North Holland Blue), obtained from the State Poultry Institute at Beekbergen. The individual weights of the animals varied between 440 and 1050 gms. In the laboratory they were housed mostly in separate cages. In the morning they were fed a diet containing 50 % wheatmeal, 25 % oatmeal and 25 % barleymeal. In the afternoon they had mixed grains. Water was given ad libitum. Twenty-five μc NaI^{131} (N.V. Philips-Roxane) was injected in a sterile and physiological solution. If the animals were treated with antithyroid drugs, these also were administered by injection. Radioiodine was injected in the left, antithyroid drugs in the right flight muscle.

At each point of time which was studied a group of 5 animals was used (in some experiments 3 animals in a group). They were weighed, just before they were killed (3–5 min., constant in each experiment) with chloroform.
The thyroid glands were removed and quickly weighed. Sometimes the individual weight of the thyroid glands of each animal was determined, sometimes however the thyroid glands of a group were pooled before weighing. Usually also a blood sample was taken to determine the radioactivity of the serum.

Next the thyroid glands of each group were pooled, homogenized and extracted 3 times in a total of 5 ml of an icecold ammonia buffer (pH $\pm$ 8.4) (Gross and Pitt-Rivers 1953). The supernatant fluids, after centrifugation, were sampled. The residue left contained hardly any radioactivity. Of the homogenate 0.1 ml was plated and the radioactivity of this sample was determined with the help of a β-counter. This enabled the calculation of the total radioactivity fixed in the glands.

Next, about 2 ml was added to 10–12 mg pancreatinum purum (Merck) in 0.5 ml of the above mentioned buffer-solution. This material then was hydrolysed for 3 days at 38° C under toluene, after which it was chromatographed, to determine the I^{131} in the different fractions.

Moreover, part of the thyroid homogenate was directly chromatographed. Sometimes 1 ml was treated with 0.5 ml of a 10% (or 20%) trichloroacetic acid solution, after which the residue was treated twice with 0.5 ml of a 2 ½% (10%) solution of trichloroacetic acid. The liquid obtained was, after neutralizing, plated and counted, sometimes also chromatographed. With the aid of this technique the amount of inorganic iodine could be ascertained, as the organically bound iodine (thyroglobulin) is precipitated by the trichloroacetic acid.

In the chromatographical procedure a small amount of the material was analysed by paperchromatography, using the full width of a 7 cm strip Whatman No 1 paper. The amount which was applied had to be rather low, as otherwise irregularities in the chromatograms (tailing) would arise from the salts from the buffer-solution.

The material was chromatographed, either with the help of a mixture of butanol, ammonia and dioxane (Gross and Pitt-Rivers 1953), or of a mixture of butanol and acetic acid (but.: 78, acetic acid: 5, aq. dest: 17).

The papers being conditioned for 4 hours, the chromatographical procedure was allowed for about 14 hours, in a room with a constant temperature of 21° C, in glass

jars with a stainless steel support, which enabled the treatment of several papers at the same time.

In preliminary experiments synthetic iodinated amino acids were chromatographed and the papers were sprayed with a solution of ninhydrin, to locate as exactly as possible the position of the different iodinated amino acids.

After the chromatographical procedure the papers were dried in a stream of warm air (temp. about 40–50° C). A strip of 3 cm (of the middle of the chromatogram) was used for counting and autoradiography. The radioactivity of each 0.5 cm was counted with the aid of a β-counter, and the results were drawn in diagrams. These diagrams were cut out. By weighing of the different peaks of the diagrams the percentage of the radioiodine in the different amino acids could be determined. As the total radioactivity in the glands was known (calculated from the mentioned 0.1 ml samples), the amount of radioiodine, present in the different fractions, could be expressed as a percentage of the dose injected, and thus the amounts of radioiodine in the same fraction after different periods of time could be compared.

In a number of preliminary experiments another method was employed. First the hydrolysis was performed with trypsinum purum, after which a rather high amount remained unhydrolysed. The iodinated amino acids then were extracted from the buffer solution by treatment with n-butanol. The n-butanol (first an acid solution, and after that a neutral solution was used) was reduced by evaporation to dryness and the residue was taken up by NaOH and chromatographed.

This method afterwards was abandoned for two reasons: First it was demonstrated that, during the butanol extraction and the evaporation, a considerable quantity of iodine was in the form of elementary iodine, probably brought about by deiodination of amino acids.

Secondly the impression was obtained that the percentage of the different iodinated amino acids after the treatment with butanol was not the same as before (by preferential solution of these amino acids in the butanol).

Therefore all experiments, quoted in this paper, were carried out with the technique described, which shows some advantages:

a. The material is almost wholly hydrolysed by the pancreatinum (in the greater part of the cases less than 5% of the total radioactivity remained at the start of the chromatograms. This compound was not analysed. When sometimes some material moved over a small distance, it is assumed to be partly hydrolysed material[1]).

b. In the countings of the chromatograms developed in butanol–ammonia–dioxane little or no radioactivity could be detected at the front (where the elementary iodine, if present, should have been found), whereas no radioactivity at all was demonstrated in the radioautograms. It is difficult to say the same for the chromatograms, developed with the help of butanol and acetic acid, as here the iodothyronines approach the front rather nearly. As the results of the percentages of the fractions in both chromatograms were quite comparable, the assumption seems justifiable that in the butanol–acetic acid chromatograms also the amount of I^{131} in the form of elementary iodine must have been very small.

c. In this method the number of the operations is reduced to a minimum. The thyroids were only homogenised and hydrolysed.

As mentioned before, the paper strips were submitted to autoradiography. The autoradiograms were prepared with the aid of Kodak-X-film.

Usually the exposure time was 2–3 weeks. As an endeavour was made to apply fairly equal amounts of material on different strips and the time of autography

[1] The possibility of the presence of some iodinated amino acids which do not move in the solutions used remains open and has to be studied (Roche, Michel, Nunez and Wolff 1955; Roche, Michel, Wolff and Nunez 1956).

was constant in each group, mostly the autoradiograms are comparable on a quantitative scale.

In a single experiment the serum (after extraction with butanol) was applied to chromatographical analysis. Extraction with butanol was needed here, as the amounts of radioactive compounds in the serum are too small to enable the direct chromatography, used in the analysis of thyroid homogenates. Moreover the addition of synthetic amino acids was necessary, as the amounts of iodinated amino acids were very small.

IV. THE SYNTHESIS OF HORMONES IN THE THYROID GLANDS OF NORMAL COCKERELS

1. *Introduction*

The technique used is based upon the demonstration of disintegrating I^{131}, whether in inorganic or organic form. The amount of radioiodine administered however has to be small with respect to the amount of the non-radioactive isotope I^{127}, because otherwise, as a result of the injection, the physiological processes may be changed.

First we may produce some data on the amounts of iodine in the thyroid glands of some birds and mammals, studied by different authors (table 1).

TABLE 1

The relative content of iodine in the thyroid glands of birds and mammals (iodine in g% in desiccated thyroid tissue)

Duck	0.862 (Elmer 1938)
Chicken	0.887 (Elmer 1938)
Ox	0.03, 0.64, 0.72, 0.45 (Michel 1950)
Horse	0.40, 0.34 (Michel 1950)
Pig	0.40, 0.08, 0.59, 0.47, 0.33, 0.21, 0.18, 0.21, 0.31, 0.32, 0.39 (Michel 1950)

From table 1 we obtain the impression that the content of iodine in the glands of birds is higher as compared to mammals.

Mr. G. de Vries (Dept. of Chemistry, Free University, Amsterdam), who kindly studied the content of iodine in some thyroid glands of cockerels, determined the content as 0.58% and 0.59%, which results in 35.5 to 46.0 γ iodine in the thyroid glands of one cock.

Other data can be obtained with the help of the goitre prevention test, which enables the determination of the require of Tx.

In man the amount required is 200 γ/day (Brenner, Black and Gaddie 1954), in chickens (Hoffman 1950) 1.98 γ/day/100 gms. (in 500 gms-animals this would be about 10 γ/day).

The iodine content in the thyroids of our animals will be about 40 γ. The other data suggest a daily input and output of some gammas iodine.

In the 25 μ curie NaI^{131}, which was injected, there was at most 0.1 γ iodine. Of this amount about 15% was taken up by the thyroid gland: 0.015 γ. So the amount of radioiodine used is very small as compared to the amount which circulates in the blood and the amount of $iodine^{127}$ which is present in the thyroid gland.

Moreover it is very probable that in the iodinated amino acids not all iodine atoms are radioactive, because the amount of I^{131} is so small as compared to the amount of I^{127}.

If the quantity of radioiodine administered is equally spread over the $iodine^{127}$ (which is valid only some time after the injection) the chance to find a disintegrating I^{131}-atom in one molecule of Tx is 4 times as high as to find one in a molecule of MIT. Starting from the percentage of radioiodine in MIT, the relative amounts for DIT, TITh and Tx should be divided respectively by 2, 3 and 4. We will return to this subject in the interpretation of the experiments.

2. *The total I^{131}-content in the thyroid gland at different times after the injection*

The results of some experiments are shown in figs. 1 and 2. All experiments were performed with groups of 5 animals, killed at different periods of time after an injection with NaI^{131}. Fig. 1 shows the presence of I^{131} in the thyroid glands up till 9 h. after the injection. Fig. 2 gives the radioiodine content in two experiments, which were continued up till 48 and 96 h.

Curves A (exp. I) and B (exp. II) in fig. 1 demonstrate the presence of radioiodine during the first hours following an injection with radioiodide in the two experiments, which were continued up to 48 and 96 h. of which the full results are given in fig. 2. The animals were killed and analysed respectively ¼, ½, 1, 2, 3½, 5, 8, 12, 16, 22, 28, 34, 40 or 48 h. after the injection in exp. I, whereas in exp. II the abovementioned times were followed by observations at 60, 72 and 96 h. after the administration of the radioiodide. Curves C and D in fig. 1 give the values of the radioiodine content in untreated animals of exp. IV, the object of which was to study the influence of KSCN (see Chapter V) on the hormone synthesis, and of exp. VI, which was a study of the same under the influence of thiourea (Chapter VI). In these experiments the animals were killed respectively at ¼, 1, 2½, 4, 6 or 9 h. after the injection with radioiodide. Curve E in fig. 1 shows the same in the untreated animals of an experiment (Exp. VIII) to study the action of Amphenone "B" (Chapter VII). In this experiment the animals were killed respectively ¼, ½, 1, 2, 3½ or 5 h. after the administration of the radioiodine.

The following notes apply to figs. 1 and 2.

1. The picture of each single curve is fairly regular. Generally the value of the iodine content at a given point of time is supported by the

foregoing and the following values. We will return afterwards to the significance of the aberrations.

In the 5 experiments quoted, the uptake of the radioiodine obviously varies. This variation may be caused by the following phenomena:

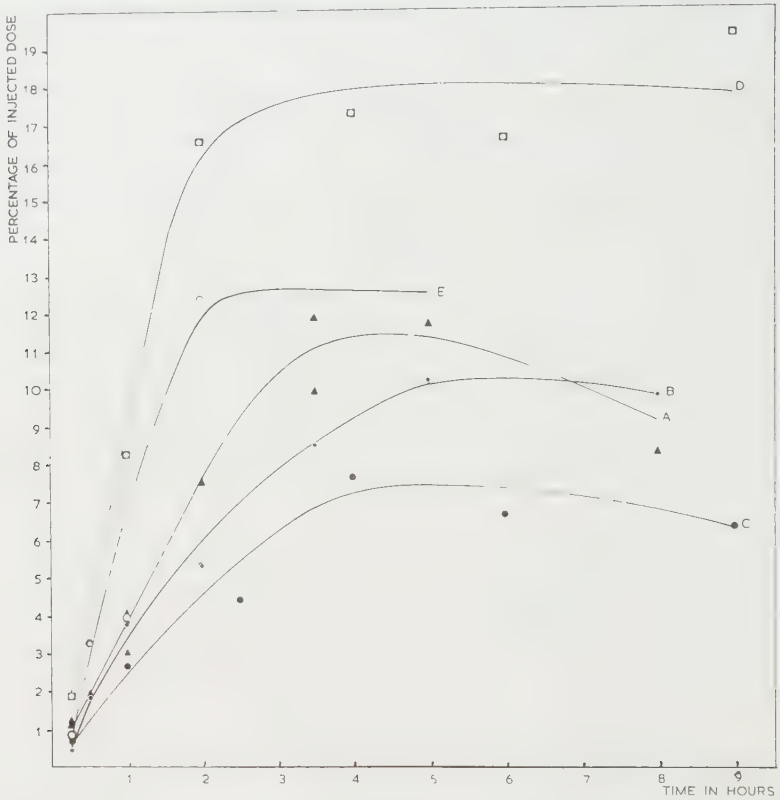

Fig. 1. Content of total radioiodine in the thyroid glands of cockerels in percentage of injected dose, up till 9 h. after the injection.

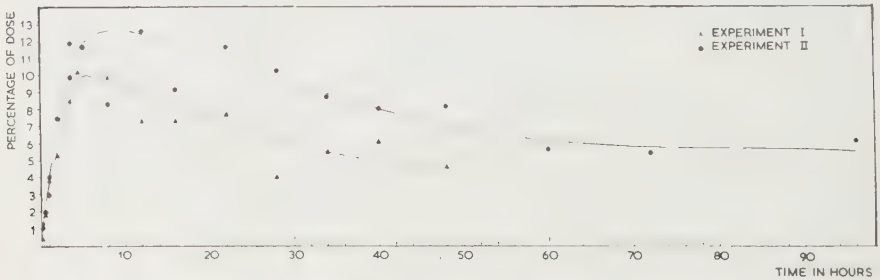

Fig. 2. Content of total radioiodine in the thyroid glands of cockerels in percentage of injected dose, up till 96 h. after the injection.

First, apart from seasonal divergences, rather fast changes in the physiological activity of the thyroid gland may occur in consequence of rises or falls in temperature.

Secondly, dietary differences in the breeding of the cockerels may have caused the variation, and so on.

2. At first the uptake of radioiodine studied in rather small intervals of time is nearly constant. Then, however, at a different time in each experiment, a gradual decrease occurs. Evidently the content of radioiodine in the thyroid gland reaches a maximum, after which the radioactivity in the gland decreases. The maximum in our experiments was attained between 2–8 hours after the injection with I^{131}. The occurrence of a maximum is the result of two simultaneous processes, the uptake and the release of radioiodine, in the beginning the first prevailing, later on the second.

The uptake of radioiodine in the thyroid gland is reciprocally related to the decrease of radioiodine in the serum. When the thyroid gland concentrates most of the iodide (in the first hours after the injection), there is a marked decrease in the serum. This relation is clearly demonstrated by table 2. In this experiment ¼, 1 and 3½ h. after an injection with radioiodide respectively, two groups of 5 animals were killed at the same time (Exp. II).

TABLE II

Relation between the amount of radioactivity in the thyroid gland and in the serum

Time in hours after injection	Radioactivity in the thyroid in percentage of injected dose	Radioactivity in 0.1 ml serum in counts/min.
¼	1.2	1485
¼	1.1	1606
1	4.0	1160
1	3.0	1695
3½	11.9	330
3½	9.9	451

From table 2 it is evident that in those groups, which concentrated more iodide in the thyroid gland, as compared to the others of the same time, the circulating blood contains less radioactivity.

3. In figs. 1 and 2, notwithstanding the fact that we pooled the thyroid glands of 5 animals, some irregularities in the content of iodine in succeeding times are apparent. These irregularities most likely are of no physiological significance, as even in groups, killed at the same time, rather strong differences in the iodine content exist, which may rise to 20–25% of the total amount of radioiodine present in the glands (see table II).

4. Finally fig. 2 shows that the biological half time of the radioiodine in the thyroid gland (i.e. the time which elapses before half the iodine of the maximum has vanished), is comparable in the two experiments cited here. This half time is respectively 35 and 48 h. It may be concluded that in normal cockerels after about 1 ½–2 days half the amount of iodine in the thyroid gland has been replaced.

In conclusion: the uptake of radioiodine in the thyroid gland in the beginning is a rather constant process. In 3 ½–8 h. the maximum has been attained. Then the content of radioiodine gradually diminishes, half of the maximum content being still present 1 ½ to 2 days after the injection. Moreover the content of the radiodiodine within the thyroid gland happens to be nearly correlated with the iodine concentration in the blood. Though the observations were done by pooling 5 animals, obviously a rather high variability in the uptake exists, causing irregularities in the picture of the uptake in time.

3. *The partition of the radioiodine among the different fractions*

To gain an insight into the physiological processes, which are involved in the production of hormones in the thyroid gland in cockerels, in Exps. 1

TABLE III

Content of total radioiodine in the thyroid glands at different times after the injection with radioiodide

Time after injection of Na^{131} in hours	Concentration in percentage of injected dose		Concentration per mg thyroid tissue in percentage of injected dose	
	Exp. 1	Exp. II	Exp. 1	Exp. II
¼	0.41	1.1	0.003	0.011
¼		1.2		0.012
½	1.8	1.9	0.016	0.016
1	3.8	3.0	0.026	0.026
1		4.0		0.034
2	5.3	7.5	0.042	0.078
3 ½	8.5	11.9	0.066	0.120
3 ½		9.9		0.103
5	10.2	11.7	0.107	0.099
8	9.8	8.3	0.091	0.089
12	7.3	12.6	0.058	0.118
16	7.3	9.1	0.062	0.080
22	7.8	11.6	0.071	0.095
28	4.0	10.2	0.038	0.079
34	5.5	8.7	0.051	0.076
40	6.1	8.0	0.054	0.082
48	4.6	8.1	0.039	0.074
60		5.6		0.056
72		5.4		0.046
96		6.1		0.056

TABLE IV

Radioiodine in the different thyroid fractions, in percentage of injected dose[1]

Experiment I

Time after injection of NaI^{131} in hours	Iodide	MIT	DIT	MIT DIT	DITh	TITh	Tx	Total of Iodothyronines
¼	0.06	0.26	0.09	0.35				
½	0.18	0.98	0.59	1.57			0.05	0.05
1	0.37	2.00	1.10	3.09		0.05	0.29	0.34
2	0.61	2.63	1.72	4.35		0.05	0.29	0.34
3 ½	1.05	4.20	2.49	6.69		0.18	0.58	0.76
5	1.07	4.57	3.27	7.84	0.06	0.16	1.07	1.29
8	1.14	4.35	3.09	7.44		0.22	1.00	1.22
12	0.64	2.84	2.61	5.45		0.07	1.14	1.21
16	0.80	2.83	2.42	5.25	0.14	0.11	1.00	1.25
22	0.86	2.57	2.99	5.56	0.15	0.15	1.08	1.38
28	0.50	1.16	1.41	2.57	0.08	0.08	0.77	0.93
34	0.45	1.96	1.94	3.90	0.10	0.10	0.95	1.15
40	0.76	2.03	2.32	4.35	0.10	0.09	0.80	0.99
48	0.58	1.47	1.93	3.40	0.08	0.08	0.46	0.62

[1] This table was obtained by using the ratio MIT/DIT from the chromatograms developed in butanol and acetic acid and calculating this ratio in the total amount found for MIT ± DIT in the chromatograms developed with the aid of a mixture of butanol-dioxane and ammonia. The total amounts of MIT ± DIT in both analyses were quite comparable.

and II the thyroid material was hydrolysed and subsequently analysed by paperchromatography (Chapter III).

In Exp. I, 70 cockerels, 8 weeks old, were used. The individual weight varied between 545 and 780 gms, the weight of the groups varying between 3105 and 3450 gms. The weights of the pooled thyroid glands of the groups varied between 94.6 and 142.6 mgs. The animals were injected with Na I^{131}, and killed at several times after the injection as mentioned before.

In Exp. II 100 cockerels, of an age of 8 weeks, were used. The individual weights varied between 550 and 855 gms, the weights of the groups (each of 5 animals) amounted 3215 to 3540 gms. The total weights of the thyroid glands of the groups varied between 90.3 mg and 116.7 mgs. The animals in both experiments were treated in the usual way (Chapter III).

First it may be noted that the uptake in the two experiments was not quite the same. Fig. 2 and table III show that perhaps some difference exists in the time of maximum uptake in both experiments. The principal characteristics however are the same.

As Plate I figs. 1 and 2 reveal (obtained with the aid of the paperchromatographical analysis in Exp. II) the following iodinated compounds were demonstrated: iodide, thyroglobulin (in a very small

TABLE V

Radioiodine in the different thyroid fractions, in percentage of concentrated dose

Experiment I

Time after injection of NaI^{131} in hours	Iodide	MIT	DIT	MIT DIT	DITh	TITh	Tx	Total of Iodothyronines
¼	14.5	63.2	22.3	85.5				
½	10.2	54.4	32.4	86.8			3.0	3.0
1	9.9	52.4	28.6	81.0		1.4	7.7	9.1
2	11.4	49.7	32.6	82.3		0.9	5.4	6.3
3 ½	12.3	49.5	29.2	78.7		2.1	6.9	9.0
5	9.9	45.5	32.6	78.1	0.6	1.5	9.9	12.0
8	11.9	44.1	31.4	75.4		2.3	10.4	12.7
12	8.9	38.7	35.8	74.5		1.0	15.6	16.6
16	11.0	38.5	33.3	71.8	1.9	1.5	13.8	17.2
22	11.1	33.0	38.2	71.2	1.9	1.9	13.9	17.7
28	12.5	28.9	35.2	64.1	2.1	2.1	19.2	23.4
34	8.3	35.6	35.2	70.8	1.9	1.9	17.1	20.9
40	12.4	33.4	38.1	71.5	1.6	1.4	13.1	16.1
48	12.6	32.0	41.9	73.9	1.8	1.7	10.0	13.5

TABLE VI

Radioiodine in the different thyroid fractions, in percentage of injected dose

Experiment II

Time after injection of NaI^{131} in hours	Iodide	MIT	DIT	MIT DIT	DITh	TITh	Tx	Total of Iodothyronines
¼	0.12	0.66	0.31	0.97				0.01
¼	0.10	0.78	0.30	1.08				0.02
½	0.18	0.95	0.68	1.63	0.02	0.06	0.01	0.09
1	0.23	1.41	1.14	2.55	0.05	0.10	0.07	0.22
1	0.26	2.04	1.52	3.56		0.14	0.04	0.18
2	0.91	4.15	2.04	6.19	0.05	0.22	0.13	0.40
3 ½	0.94	6.64	3.26	9.90	0.19	0.49	0.38	1.06
3 ½	0.94	5.09	2.96	8.05	0.13	0.49	0.29	0.91
5	1.52	4.46	3.31	7.77	0.23	0.34	1.84	2.41
8	0.75	3.41	2.76	6.17	0.12	0.15	1.11	1.38
12	2.09	4.46	3.40	7.86	0.15	0.38	2.12	2.65
16	0.66	3.01	3.11	6.12	0.08	0.25	1.99	2.32
22	0.99	3.66	4.18	7.84	0.16	0.20	2.41	2.77
28	0.92	3.30	3.65	6.95	0.21	0.30	1.82	2.33
34	1.27	2.02	3.55	5.57	0.10	0.18	1.58	1.86
40	0.81	2.20	2.76	4.96	0.15	0.24	1.84	2.23
48	0.62	2.29	3.40	5.69	0.10	0.21	1.48	1.79
60	0.60	1.50	2.23	3.73	0.10	0.17	1.00	1.27
72	0.81	1.15	2.18	3.33	0.09	0.11	1.06	1.26
96	0.65	1.43	3.05	4.48	0.07	0.08	0.82	0.97

TABLE VII

Radioiodine in the different thyroid fractions, in percentage of concentrated dose

Experiment II

Time after injection of NaI^{131} in hours	Iodide	MIT	DIT	MIT DIT	DITh	TITh	Tx	Total of Iodothyronines
¼	10.9	59.4	28.4	87.8		1.3		1.3
¼	8.1	65.0	23.9	89.9		2.0		2.0
½	9.6	49.6	34.3	84.9	1.3	3.0	1.2	5.5
1	7.6	47.1	37.9	85.0	1.6	3.5	2.3	7.4
1	6.4	50.9	38.0	88.9		3.6	1.1	4.7
2	12.1	55.3	27.2	82.5	0.7	2.9	1.8	4.4
3 ½	7.9	52.6	30.6	83.2	1.6	4.1	3.2	8.9
3 ½	9.5	51.6	29.6	81.2	1.3	5.0	3.0	9.3
5	13.0	38.1	28.3	66.4	2.0	2.9	15.7	20.6
8	9.0	41.2	33.2	74.4	1.5	1.8	13.3	16.6
12	16.6	35.4	27.0	62.4	1.2	3.0	16.8	21.0
16	7.3	33.1	34.2	67.3	0.9	2.8	21.7	25.4
22	8.5	32.5	35.1	67.6	1.4	1.7	20.8	23.9
28	9.1	32.4	35.7	68.1	2.1	2.9	17.8	22.8
34	14.6	23.2	40.8	64.0	1.3	1.9	18.2	21.4
40	10.1	27.5	34.5	62.0	1.9	3.0	23.0	27.9
48	7.6	28.2	42.0	70.2	1.3	2.6	18.3	22.2
60	10.8	27.8	39.8	66.6	1.8	3.0	17.8	22.6
72	15.0	21.3	40.4	61.7	1.7	2.0	19.6	23.3
96	10.7	23.5	50.0	73.5	1.1	1.3	13.4	15.8

amount which persists after hydrolysis), MIT, 3 : 5 -DIT, 3 : 5 -DITh, 3 : 5 : 3′ -TITh and Tx, moreover some material, which is assumed to be some partly hydrolysed material.

The results of the paperchromatographical analysis are (beside Plate I figs. 1 and 2 which show the autoradiograms of Exp. II) communicated in tables IV, V, VI and VII, and in the figs. 3, 4, 5 and 6.

These tables and figs. show the absolute (in percentage of injected dose) and relative amounts of I^{131} in the different fractions. In Exp. I the amounts of 3 : 5 -DITh and 3 : 5 : 3′ -TITh were very low; in the figs. they are shown together with Tx.

These results allow the following comments to be made:

I^{131} in MIT (figs. 3, 4, 5 and 6; tables IV and VI).

Especially during the first experimental hours, the I^{131} within the thyroid gland is found in this fraction. The curve of MIT with time shows great resemblance to the curve of the total radioiodine in the gland. After a maximum the amount of radioiodine in MIT decreases (figs. 3 and 4). The highest amount of radioiodine in this fraction is present up till about 18 h. after the injection. From the start of the experiments the percentage of radioiodine in this fraction diminishes (figs.

5 and 6). During the first 3½ h. the percentage is 50–60%, in the time following decreasing, so that 24–48 h. after the injection about 25–35% of the total radioiodine is in the form of MIT.

I^{131} in DIT (figs. 3, 4, 5 and 6; tables IV and VI).

In this fraction also the radioiodine is found at the very beginning of the experiments, though in smaller quantities than in MIT. The maximum content of radioiodine in this fraction also is reached later than in MIT. Afterwards it decreases, but to a lesser extent as compared to the radioiodine in MIT. In the relative quantities however the I^{131} in DIT increases from 25% at the start of the experiment to about 40% at the later times of observation.

I^{131} in Tx (figs 3, 4, 5 and 6; tables IV and VI).

In the beginning radioiodine is found in this fraction in very small amounts. However it increases gradually to reach a maximum between 10 and 20 h. after the injection.

The relative amount of iodine in this fraction is highest in Exp. I at about 30 h. after the injection, in Exp. II it remains at a level from about 16 up to 72 h. (tables V and VII).

I^{131} in 3 : 5 : 3′ – TITh (figs. 4 and 6; tables IV and VI).

Small quantities of radioiodine are shown in this fraction (mostly less

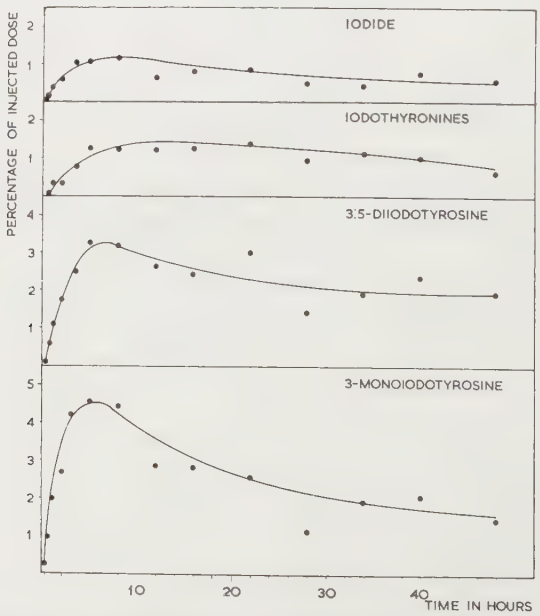

Fig. 3. Radioiodine in the different thyroid fractions, in percentage of injected dose (Exp. I).

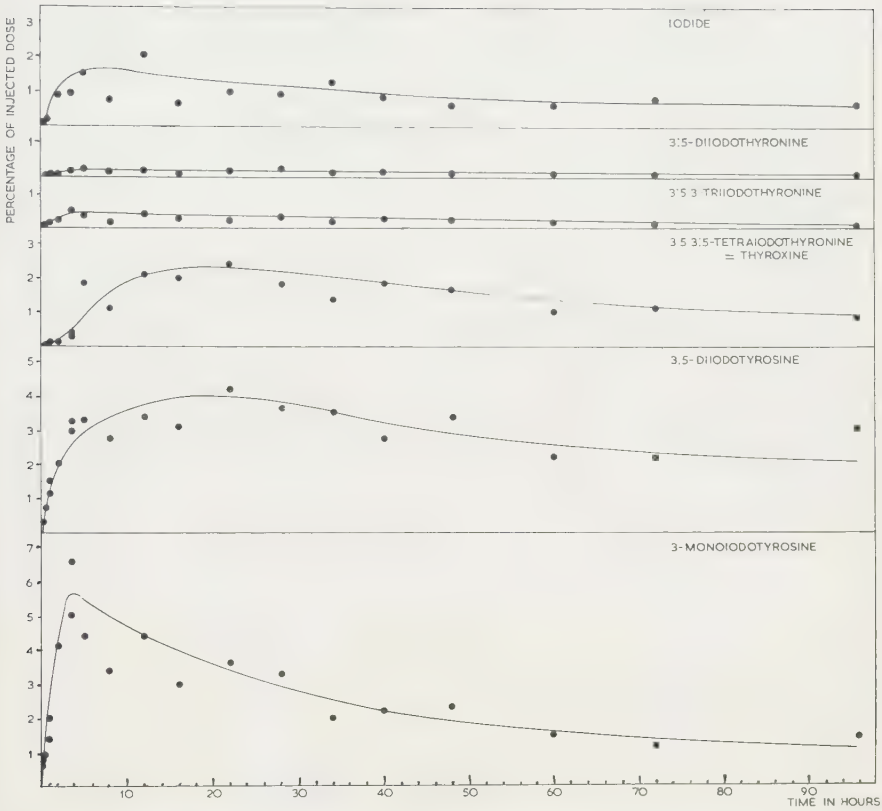

Fig. 4. Radioiodine in the different thyroid fractions, in percentage of injected dose (Exp. II).

than 5% of the total content of I^{131}). In Exp. II, which was most exact, a maximum appears 3½–5 h. after the injection, both relatively and absolutely, whereas after that it is found in small amounts (tables VI and VII).

I^{131} in 3:5 – DITh (figs. 4 and 6; tables IV and VI).
Rather constantly in this compound radioiodine is demonstrated in minute amounts.

I^{131} in iodide (figs. 3, 4, 5 and 6).
Both relatively and absolutely the radioiodine in this fraction shows rather important variations. The amounts range between 7 and 15% of the concentrated radioactivity.

The impression is obtained that the amounts at the start of the experiments are somewhat higher than afterwards.

From these results it is evident that the iodide, which is concentrated within the thyroid gland (see Chapter VI, and Chapter VII), as elementary iodine is bound fast to the amino acids. Some results, which are not quoted here, revealed that 5 min. after an injection, the greater part of the radioiodine is in organic form. In the first instance this organically bound radioiodine is found mainly in MIT. As is generally supposed, this compound thus has to be a first phase in the synthesis of the hormones.

Beside this, immediately after the injection increasing amounts of radioiodine are found in DIT. The amount of radioiodine in this fraction however, hardly rises above the amount in MIT (not until about 20 h. after the injection). As DIT has two iodine atoms, whereas MIT has only one, the possibility of detecting an I^{131}-atom in DIT is twice as high as for MIT. From this it is concluded that the amount of DIT is low as compared to MIT. On the other hand the amount of Tx (also with regard to the molecules) is low as compared to that of DIT.

However, we have to realise that in the successive processes in the

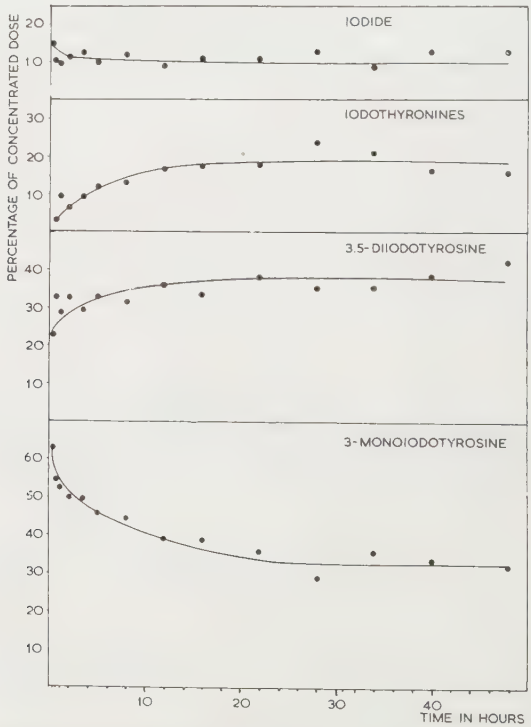

Fig. 5. Radioiodine in the different thyroid fractions, in percentage of concentrated dose (Exp. 1).

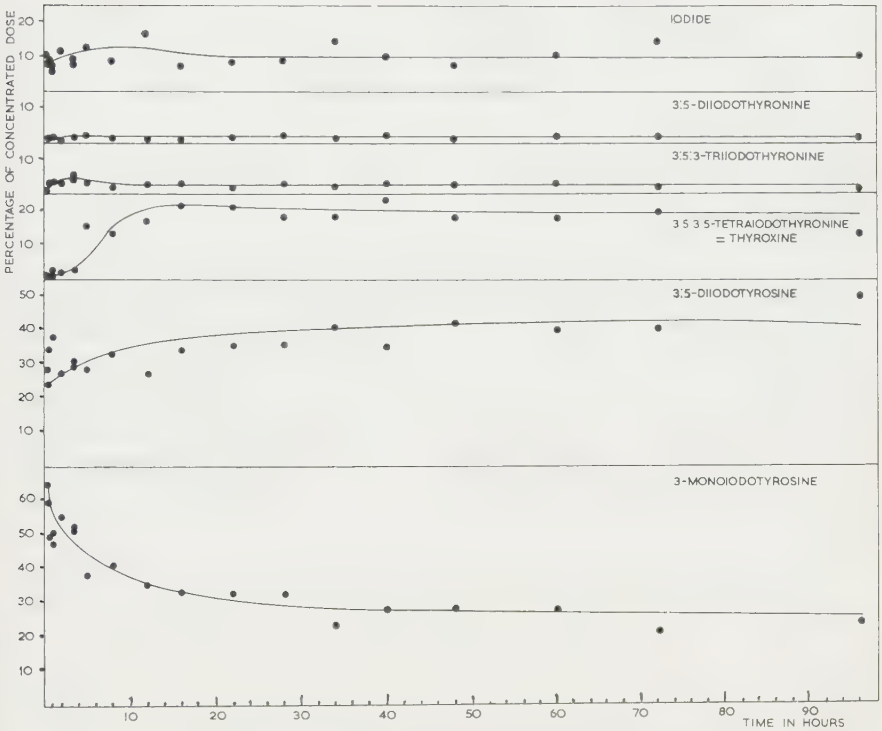

Fig. 6. Radioiodine in the different thyroid fractions, in percentage of concentrated dose (Exp. II).

thyroid gland an increasing concentration of the iodine in the individual molecules takes place, so that the molecules of the eventual products (Tx and TITh, to a less extent also DITh) exist in relatively small numbers, but they have a great part of the iodine.

Going further into the iodothyronines, it is evident that there exists a fairly clear connection between the curves of the iodothyronines and those of the iodotyrosines.

It is shown in Exp. II that the curve of Tx ressembles that of DIT (both attain their maximal value at about the same time). This must be the consequence of the origin of Tx (the most important iodothyronine) by condensation of two molecules DIT.

On the contrary the 3 : 5 : 3′-TITh (fig. 4) shows a strong analogy with MIT, which supports the idea that TITh would originate by condensation of one molecule MIT and one molecule DIT. We return to this point in the discussion of the results. Nevertheless it may be mentioned here, that the origin of 3 : 5 : 3′-TITh before that of Tx, contradicts the origin of 3 : 5 : 3′-TITh by deiodination of Tx.

With respect to the 3 : 5-DITh only general remarks can be given. The amounts of the radioiodine in this fraction were rather small, no peaks being observed. This allows no definite conclusions. We will come back to this point in the discussion of the results.

Tables IV and VI, and figs. 3 and 4 indicate rather strong variations in the amounts of the radioiodide. To study the meaning of this, we tried also to determine the radioiodide with the help of the trichloroacetic acid method (T.C.A.), which enables (after TAUROG, TONG and CHAIKOFF 1950, and WIJNGAARDEN, WRIGHT, and WAYS 1952) the determination of the inorganic iodine, by precipitating the organically bound iodine. The initial preparations, appeared to have a considerable amount of thyroglobulin, when analysed by paperchromatography. Merely in Exp. II, with a changed technique (see Chapter III) the results obtained were reliable. For this reason in table VIII the amounts of radioiodide, determined by chromatographical analysis, are compared with those got by the T.C.A.-method. Moreover the ratio between the two has been calculated.

The data show that the amounts of iodide, determined with the T.C.A.-method evidently are lower than those obtained with the paperchroma-

TABLE VIII

Amount of radioiodide in the thyroid gland, in percentage of injected dose, as determined by paperchromatographical analysis and by the T.C.A.-method and the quotient between the two determinations (Exp. II)

Time after injection of NaI^{131} in hours	Amount of radioiodide (chromatographical analysis)	Amount of radioiodide (TCA-method)	Quotient beween the two determinations
¼	0.12	0.05	2.4
¼	0.10	0.05	2.0
½	0.18	0.10	1.8
1	0.23	0.12	1.9
1	0.26	0.22	1.1
2	0.91	0.13	7.0
3 ½	0.94	0.37	2.5
3 ½	0.94	0.40	2.3
5	1.52	0.37	4.1
8	0.75	0.32	2.3
12	2.09	0.32	6.5
16	0.66	0.46	1.5
22	0.99	0.48	2.1
28	0.92	0.28	3.3
34	1.27	0.36	3.5
40	0.81	0.35	2.3
48	0.62	0.31	2.0
60	0.60	0.22	2.8
72	0.81	0.25	3.2
96	0.65	0.28	2.3

tographical analysis. Apparently the relative amounts of radioiodide, as obtained by the T.C.A.-method, vary between 1 and 5% of the total content of radioiodine in the glands.

Possibly the differences between the two methods are caused by the following factors:

First it is imaginable that these differences are caused by the technique. The iodide possibly may be partly precipitated with the protein, when treated with T.C.A., as it possibly is adsorbed to it. If hydrolysed however, it would be liberated from the protein, as by hydrolysis adsorptive bindings will disappear, so in the chromatographical analysis all iodide may be demonstrated. In connection with this hypothesis the paper of HALMI and STUELKE (1956) is very interesting. They concluded that in P.T.U.-treated rats some of the radioiodide concentrated in the thyroid gland after injection, could not be discharged with $NaClO_4$. Perhaps the difference in the iodide value between the two methods applied in our study, is also partly caused by the fact that some of the iodide is non-dischargable. Nevertheless the variety in the values at the different times remains unaccountable. (See also HALMI, in WOLSTENHOLME and MILLAR 1957.)

On the other hand the possibility exists that, during the chromatography, deiodination of iodinated amino acids causes an increase in the amount of iodide. This possibility is supported by the fact that the quotient of the two values obtained is high at the times which showed high amounts of iodide according to the paperchromatographical analysis (table VIII). If deiodination occurs, it is probable this deiodination will have happened especially on the account of MIT and DIT, as the amounts of 3 : 5 : 3′-TITh would have been much higher at the end of the experiment when this deiodination had happened on account of the Tx.

Possibly in this connection the deiodination, which was demonstrated by ROCHE, MICHEL and LISSITZKY (1951), and ROCHE, MICHEL, MICHEL and LISSITZKY (1953) to occur in free MIT and DIT, is of interest. Once again the variety in the values, however, remains inexplicable.

We may conclude that the experiments reveal that the processes, which are involved in the production of thyroid hormones in birds, are essentially the same as those demonstrated in mammals (see Chapter II).

First the iodide is concentrated in the thyroid gland. This iodide is – after oxidation, presumably by an enzyme system – bound to amino acids. This binding results in the origin of MIT. At the same time 3 : 5-DIT, first in lesser amounts, comes into existence. By condensation of MIT and 3 : 5-DIT also 3 : 5:3′-TITh originates. This compound

shows its maximum at the time that the amount of MIT is maximal. The amounts of 3 : 5 : 3′-TITh however are small. Afterwards, in much higher amounts, Tx comes into being by condensation of two molecules of 3 : 5-DIT. It has a maximum at the time in which maximal amounts of the forementioned compound are present.

4. *The relative amounts of the radioiodine in the different fractions*

As a further analysis of the processes in the thyroid glands of birds, the relative amounts of the different fractions are compared (figs. 5, 6).

First the transformations in the relative amounts are to be studied. As mentioned before, the content of radioiodide in MIT originally is very high (60%), afterwards it decreases gradually, whereas the initial content in 3 : 5-DIT is low (20–30%) and afterwards increases. These phenomena partly are dependent on each other, and also, as was shown, on the origin of iodinated thyronines. Therefore, to get an insight about the progresses in the formation of MIT and DIT it will be better to account for the relations of the I^{131} in these two compounds. The quotients were calculated for the Exp. I and II (table IX).

TABLE IX

The value of the quotient I^{131} in MIT/I^{131} in DIT in Exp. I and Exp. II

Time in hours after the inj.	I^{131} MIT/I^{131} DIT Exp. I	Exp. II
¼	2.9	2.1 2.6
½	1.8	1.4
1	1.7	1.2 1.3
2	1.5	2.0
3½	1.7	2.0 1.7
5	1.4	1.3
8	1.4	1.2
12	1.1	1.3
16	1.2	1.0
22	0.9	0.9
28	0.8	0.9
34	1.0	0.6
40	0.9	0.8
48	0.8	0.7

From these data, affirmed by other experiments, it is evident that in a small period following the injection most of the radioiodine is found in MIT. In consequence the quotient has a high value. This value however decreases rapidly during the next hours (in most experiments one hour after the injection the value is about 1.5). Afterwards the decrease is more gradual. After about 16 – 22 h. the quantities of radioiodine in

MIT and DIT are the same. In the next period the value falls below 1, as the amount of I^{131} in DIT now is higher than in MIT. It may be assumed, though the data are not very conclusive on the point, that a constancy in the quotient will be reached.

In the interpretation of these data it has to be mentioned first that TAUROG, TONG and CHAIKOFF (1950) demonstrated a constant relation between the percentages of I^{127} in the different amino acids in the thyroids of rats. This relation was proved to be independent of the total iodine content within the glands. ROCHE and MICHEL (1956) also agree with this conception. So the decrease in MIT/DIT must be caused by the passage of the small quantity I^{131} along the different steps involved in the production of the hormones. Furthermore it is clear that the radioactive DIT which was demonstrable is formed in different ways: 1. by iodination of radioinactive MIT with I^{131} (which happens especially in the beginning of the experiments); 2. by iodination of radioactive MIT with I^{127} (increasing according as more radioactive MIT is synthesized). In these ways DIT with 1 atom I^{131} is synthesized; 3. DIT with 2 atoms I^{131} will originate in negligible quantities, as the amount of I^{131} is very small in relation to the amount of I^{127}. Therefore we may exclude this possibility from our considerations. The fast decrease of MIT/DIT during the first hours of the experiments proceeds from the fact that DIT not only originates from radioinactive MIT, but also from radioactive MIT.

As an introduction to the discussion of the processes involved in the synthesis of the thyroid hormones, it may be noticed that from the curves of the total I^{131}-content in the thyroid gland it is evident that after a maximum, a continuous decrease of radioiodine sets in. This must be the result of the release of thyroid hormones (especially Tx) into the blood. This release however is preceded by proteolysis of the thyroglobulin. In this enzymatic hydrolysis, beside the iodothyronines, the iodotyrosines are set free. These tyrosines – precursors of the iodothyronines – are demonstrable in thyroid homogenates. In Plate II fig. 1 a reproduction is given of the autoradiographical results of *un*hydrolysed thyroid material at several times after the injection with radioiodide (Exp. II). However, the free MIT + DIT, shown here, in the thyroid gland, are not found in the blood (Plate II fig. 2). Therefore probably they are dehalogenated by deiodinating enzymes within the thyroid gland (ROCHE, MICHEL, MICHEL, LISSITZKY 1952). Once again the radioiodine is used for the iodination of amino acids in the thyroglobulin. Only the radioiodine which is bound to iodothyronines disappears from the thyroid circulation. If we consider that at most 30% of the radioiodine is present in the form of iodothyronines (table V and VII) and that about half of the concentrated radioiodine is released

in 1 ½–2 days from the glands, the importance of the hydrolysis of the thyroglobulin is clearly demonstrated. At best ⅓ of the iodine atoms passes the thyroid cycle once, the left twice or more.

The maximum in the iodothyronines at about 20 h. (10–12 h. after the maximal iodine content in the thyroid gland), as well as the importance of the deiodination mentioned above, both suggest that at about this time (20 h. after the inj. in Exp. II) the mixing of the I^{127} and I^{131} atoms must be fairly complete.

It could be expected that after a complete mixing of radioactive and radioinactive iodine atoms at about 20 h. and a maximum in the amount of iodothyronines at the same time, a rather constant level of the relative amounts of MIT and DIT would be found. Nevertheless we mentioned before that the decrease or increase in the relative amounts of MIT and DIT lasted for a longer time (up to about 30–40 h. after the injection). The one explanation we could find to approximate what happens, possibly is a difference in the rate of production of hormones in the different thyroid follicles.

Nadler, Leblond and Bogoroch (1954) demonstrated in rats that the peripheral follicles (mostly of a greater size) concentrated less I^{131}, but at the same time the biological half time of the iodine in these follicles, was markably higher than in the smaller central follicles which concentrated the I^{131} and released it very quickly. In consequence the biological half time value of the I^{131} in these follicles was much smaller.

Probably the same holds for the thyroid gland of cockerels, for in these animals the histological structure of the (much larger) peripheral follicles points to a far smaller physiological activity in comparison with the central follicles. This is in keeping with the vascularization, which is much lower in the periphery of the thyroid gland (Lever 1948, 1950). We observed that the decrease of MIT, and also the increase of DIT was asymptotic. Now, the beginning of these curves perhaps can be explained by what happens in the small central follicles, whereas the end would be caused by the large peripheral follicles, where the mixing of the iodine takes a longer time.

Furthermore the curve of the relative amounts of Tx (in Exp. I all iodothyronines are taken together, but the amount of Tx is much larger than that of the other iodothyronines) shows a striking resemblance with that of DIT. From Exp. I as well as from Exp. II the impression is obtained that a maximum in the relative amount is attained, after which the amount of Tx decreases to reach a rather constant level afterwards.

Though the data show some variations, it may be supposed that this picture (the maximum and the decrease) is caused by the small central follicles, which, as stated above, release the iodine at a high rate. A clear connection however is not observed.

From what has been said above it seems justifiable to draw the following conclusions as to the normal relative amounts of the different amino acids in comparison to each other.

By calculation the relative partition of the radioiodine in the fractions in Exp. I is shown to be for MIT, DIT and Tx 32.6, 37.3 and 18.3 respectively. In Exp. II the mean values are 27 : 39.8 : 22.5. So it seems that in normal cockerels the relative amounts of iodine in these fractions are about 30 : 38 : 20.

When taking into account that relatively a thyroninemolecule is easier demonstrable than a tyrosinemolecule (see Introduction to this Chapter), and supposing that the demonstrated iodothyronine wholly is Tx, it may be concluded that the real amounts of iodinated amino acids are about 6 : 4 : 1 for MIT, DIT and Tx (iodothyronines).

It will be interesting to study the differences in the partition of the radioiodine among the different fractions between the two experiments. First it is evident that in Exp. I less radioiodine is found within the thyroid gland than in Exp. II (fig. 2). Secondly in Exp. I an equilibrium in the partition of the radioiodine seems to occur at an earlier time after the injection than in Exp. II. Last it may be mentioned that in Exp. I the biological half time of the radioiodine in the gland is about 35 h., whereas in Exp. II it is shown to be about 48 h.

The relative amounts of the iodinated amino acids are shown in table X.

TABLE X

Relative amounts of MIT, DIT and Thyronine in Exp. I and II

	MIT	DIT	Thyron.
Exp. I	7.1	4.1	1
Exp. II	4.8	3.5	1

This table shows that the amount of iodotyrosine in relation to the amount of iodothyronine in Exp. I is higher than in Exp. II. In the first instance this may be thought to point to a lower physiological activity of the thyroid glands of Exp. I, as compared to that of Exp. II. This seems to be in keeping with the lower maximum content of radioiodine (fig. 2) in Exp. I. On further analysis however, this conclusion obviously is a misconception. A first indication of the incorrectness is shown by the fact that constancy in the relative amounts in Exp. I earlier is attained than in Exp. II. This points to a higher iodine metabolism in Exp. I.

This is also indicated by the biological half time which is shorter in Exp. I, for the biological half time of the radioiodine is supposed to

diminish dependent on a higher metabolism of the iodine. Thus, if in Exp. II the total iodine content in the gland is higher, and this is related with a higher physiological activity, this iodine has to be released at a higher rate as thyroid hormones. This definitely is not the case.

Fig. 2 shows that possibly in Exp. I the maximal content of radioiodine is attained earlier (after about 6 h.) then in Exp. II. This also would be in keeping with a higher physiological activity of the thyroid glands in Exp. I, as the curve of the total content of radioiodine is determined by uptake and release of radioiodine. The earlier occurrence of a maximum content in Exp. I then may be caused by an early prevalence of the release above the uptake of I^{131}. The higher content of radioiodine in Exp. II then is a consequence of a lower physiological activity. This is in keeping with a higher rate of the uptake of the radioiodine in the thyroid gland in Exp. II as compared to Exp. I. This also is explainable by the high rate of iodine release in Exp. I.

Lastly the relative amounts of amino acids also are in keeping with the foregoing notes. The bodies of the cockerels of Exp. I seem to have asked for more hormone in comparison with those of Exp. II. This results in a continuously higher proteolytic hydrolysis of the thyroglobulin. When formed, this will be broken down at a high rate. The time needed for the successive steps in the synthesis of the hormones is diminished and the thyroid hormones are released at a higher rate. This is the reason why the quotient radiotyrosine/radiothyroxine in this case is much higher than in Exp. II. The result of a higher proteolytic hydrolysis of the thyroglobulin is not only a relatively low amount of iodothyronines but also a prevalence of MIT over DIT.

In conclusion: in studies of the physiological activity of the thyroid gland and of the rate of the processes involved in the synthesis of the thyroid hormones, it is necessary to investigate the partition of the radioiodine among the different fractions and the biological half time of the iodine within the gland. In the case that in two different groups of thyroid glands of animals at a definite time the relative amounts of radioiodine in Tx are different, this does not mean that the glands with the highest amount of radiothyroxine have the greatest physiological activity – on the contrary, mostly a higher physiological activity is related with a lower amount of radiothyroxine.

5. *Discussion*

In this study 3 : 5 : 3′-TITh for the first time is demonstrated in the thyroid gland of birds.

As for 3 : 5-DITh conclusions may be cautiously drawn. Though Roche, Lissitzky and Michel (1951) demonstrated this compound in

the thyroid gland of rats, in only small quantities, ROCHE and MICHEL (1955) speak of a compound which, chromatographically, had the same properties as 3 : 5-DITh. It has no, or only very small physiological activity (ROCHE and MICHEL 1956). PITT-RIVERS (GROSS and PITT-RIVERS 1954) thinks the iodination of thyronine at 3 and 5 impossible. The formation of 3 : 5-DITh by condensation of 3 : 5-DIT and tyrosine is not referred to, probably indicating that the authors dissent from this possiblility. So, 3 : 5-DITh only occasionally has been detected in in thyroid glands.

As we demonstrated this compound only in minute amounts in the thyroid gland, whereas it was not found in the blood, the possibility must be open, seen the above, that this is some other compound, strongly resembling the 3 : 5-DITh.

Undoubtedly 3 : 5 : 3′-TITh exists in the thyroid gland and has physiological activity (sometimes we could demonstrate chromatographically small amounts in the serum).

Generally the amounts are small. Probably this is the reason – beside the technique – why neither STOLL and BLANQUET (1952), nor TAUROG, TONG and CHAIKOFF (1950), nor TRUNNELL and WADE (1955) were able to detect this compound in chicken embryos and adult birds. The plausibility of the origin of 3 : 5 : 3′-TITh by condensation of MIT and DIT was stressed by the results of the investigations. This is in accordance with the opinion of ROCHE, LISSITZKY and MICHEL (1953). Though initially GROSS and PITT-RIVERS (1953) claimed the origin of TITh by deiodination of Tx, afterwards they abandoned this opinion (GROSS and PITT-RIVERS 1954). The experiments cited here stress the origin by condensation of MIT and DIT (3 : 5 : 3′-TITh was demonstrated before Tx could be detected, the quantity diminished at the time that Tx in a higher quantity is synthesized). This also is in agreement with the studies of LELOUP and LACHIVER (1955). TITh may be considered as a precursor of Tx, as the amount decreases when Tx increases (see also ROCHE and MICHEL 1956). In our experiments the resemblance of the curves of MIT and TITh on the one hand, of DIT and Tx on the other hand is apparent.

With respect to the relative quantities of the different iodinated amino acids the data in the literature are rather unreliable. As for birds TAUROG and CHAIKOFF (1947) state that about 25% of the total iodine in the thyroid gland is present as Tx. This is in good agreement with our results. TAUROG, TONG and CHAIKOFF (1950) detected 20% of the iodine in MIT in birds, 15% in rats. In DIT 25 and 30% respectively was found, whereas the amount of iodine in Tx for both was about 20–25%. These data however are rather unreliable, as the authors employed an alkaline hydrolysis. ROCHE, JUTISZ, LISSITZKY and MICHEL

(1951) have shown the advantages of an enzymatic hydrolysis. Our data (MIT : DIT : Tx = 30 : 38 : 20, in which all other iodothyronines are added to Tx) undoubtedly are more reliable.

TAUROG, TONG and CHAIKOFF (1950) concluded the presence of more MIT in birds, as compared to mammals. To check this conclusion, we will compare our data with those of ROCHE, LISSITZKY and MICHEL (1953), also employing an enzymatic hydrolysis, on normal rats. Partly their results are in agreement with ours. They observed a gradual decrease in MIT, about 72 h. after an injection with radioiodine 27–28% of the total content of radioiodine being found in MIT. The amount in DIT however showed a decrease from about 40% after 10 h., to about 30% after 24–72 h. On the contrary we observed an increase in the radioiodine content of DIT. When averaging the amounts of ROCHE, LISSITZKY and MICHEL between 24 and 72 h. (supposing a constant relation between I^{131} and I^{127} for this period) and excluding their remnant activity, we can compare their results with ours in table XI.

It is interesting to include the values of BRAASCH, ALBERT, KEATING, and BLACK (1956) for man.

TABLE XI

The relative amounts of radioiodine in different iodinated amino acids

	MIT	DIT	Tx	TITh	DITh	Iodide
Rat (ROCHE *et al.*)	33	33	22	6	—	6
Man (BRAASCH *et al.*)	19	27	38	9	—	7
Cockerel	28	40	20	3	2	7

From these data it is evident that in cockerels more iodine is found in DIT, less in MIT as compared to rats. This probably is related with less 3 : 5 : 3′-TITh in birds.

We do not yet see any further consequence from these differences. When comparing the data of BRAASCH, ALBERT, KEATING, and BLACK (1956) with our results, considerable differences exist. Whereas on the one hand the ratios between MIT and DIT in man and cockerels are comparable, (1 : 1.4), on the other hand the tyrosines together in man have a much lower iodine content than in cockerels (46% in man to 68% in cockerels). On the contrary the relative amount of Tx in man is much higher (47 to 25 in cockerels).

From these data the impression is obtained that in man the thyroid gland works much more economically, compared to the cockerel. In cockerels ⅔ of the iodine is found in precursors, whereas in man almost half of the iodine is found in thyroid hormones.

With respect to our results the data of LELOUP and LACHIVER (1955) are of great interest. These investigators at different times after an injection with radioiodine, analysed the thyroid glands of rats maintained on an iodine-deficient diet, in comparison with rats on a diet rich in iodine. In the iodine-deficient rats they found, in physiologically active thyroid glands, relatively high amounts of MIT (45.0–54.4%) and TITh (5.6–9.6%), and low amounts of DIT (18.6-24.2%) and Tx (3.7–5.4%), whereas in the thyroid glands of rats on a diet, rich in iodine, – the synthesis of hormones being low here – they found low amounts of MIT (23.1–28.9%) and TITh (1.1–2.8%), and high amounts of DIT (50.0–54.0%) and Tx (3.9–11.1%).

They conclude that, combined with a rapid synthesis of hormones, especially TITh is formed, whereas when the synthesis of hormones is at a lower rate, especially Tx is synthesized.

It is interesting that in our experiments, which studied the normal synthesis of hormones, some resemblance with these results could be obtained. When the hormones are synthesized fairly quickly, more radioiodine can be found in MIT, as mentioned above. Moreover in this case less radioactivity was found in iodothyronine, as compared to a slower synthesis of hormones.

It is striking that in the experiments of LELOUP and LACHIVER the percentage of radioiodine in iodothyronines, in rats on a diet rich in iodine, increases to the end of the experiments. This suggests that in a period, which was not investigated by the authors, the relative content of radioiodine in iodothyronines would be higher in the rats on a diet rich in iodine, as compared to the iodine-deficient rats. This would support our data that, when thyroid hormones are synthesized in a high rate, we may expect a lower relative amount of radioiodine in the iodothyronines. LACHIVER and LELOUP (1955) are of opinion that the quotient radioiodine MIT/radioiodine DIT is a determinant for the quantity of TITh or Tx which is formed. (Value of the quotient high – a high amount of TITh, value of the quotient low – a high amount of Tx.) Out of our experiments – on the synthesis of hormones in normal cockerels – we obtain the impression that the quotient MIT/DIT is related to the quickness of the synthesis of hormones, and thus with the physiological activity of the thyroid gland.

We did however not observe a high amount of TITh in relation to a high value of MIT/DIT, but we could reason that if the release of iodothyronines were high, the relative amount of iodothyronine in the thyroid gland would be lower.

In discussing the problems of the synthesis of the hormones, the consequence of the proportional distribution of the I^{131} among the I^{127} often not has been realised. Yet this follows from the deiodination of

the free MIT and DIT, as demonstrated by ROCHE, MICHEL and LISSITZKY (1951) and by ROCHE, MICHEL, MICHEL, GORBMAN and LISSITZKY (1953). Especially in the older literature false conclusions have been drawn with respect to this point. Thus MORTON, PERLMANN, ANDERSON and CHAIKOFF (1942) found that the relative amount of radioiodine in DIT (which includes probably MIT) decreases up till 96 hours after the injection with radioiodine, whereas the iodine in the Tx fraction increases. This would suggest that eventually all radioiodine would be in Tx-form.

In accordance with the foregoing notes on deiodination this must be a misinterpretation in consequence of lack of experimental data. BRAASCH, ALBERT, KEATING and BLACK (1955) determined the specific activities of the iodotyrosines and of Tx. They state that at some time these specific activities should be the same. However they exclude TITh, which was demonstrated, and so their calculations in our opinion are invalid, as also TITh is synthesized by condensation of MIT and DIT, which act as precursors.

To gain an insight into the physiological activity of the thyroid gland one has to base oneself on the relative amounts of the iodinated amino acids, which, some time after an injection with radioiodide, have to reach constant levels.

Only then is it possible to obtain a notion about the physiological activity of the thyroid gland.

V. THE SYNTHESIS OF THYROID HORMONES IN KSCN-TREATED COCKERELS

1. *Literature*

BARKER, LINDBERG and WALD (1941), in patients, receiving KSCN-therapy for hypertension, noticed a hyperplasia of the thyroid gland. So KSCN seemed to have a goitrogenic activity. Initially the causes of this antithyroid effect were unclear. FRANKLIN, CHAIKOFF, and LERNER (1944) argued for a blocking action in the synthesis of hormones by KSCN, based on in vitro experiments. RAWSON, TANNHEIMER and PEACOCK (1944) however, in studies on rats, concluded that in vivo KSCN stimulated the uptake of iodine. This controversy was made up by a number of investigations. The experiments of WOLFF, CHAIKOFF, TAUROG and RUBIN (1946), of VANDERLAAN and BISSELL (1946), of VANDERLAAN and VANDERLAAN (1947), and of TAUROG, CHAIKOFF and FELLER (1947) should be mentioned here. These authors could ascertain that KSCN inhibits the concentration of iodide by the thyroid

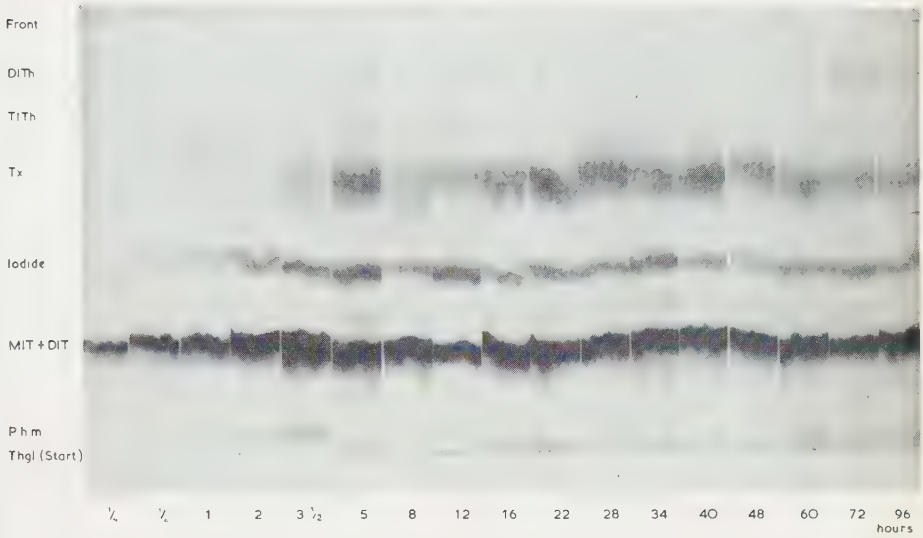

Fig. 1. Radioautograms of hydrolysed thyroid material of cockerels of Exp. II (chromatographed in but., amm., and diox.).

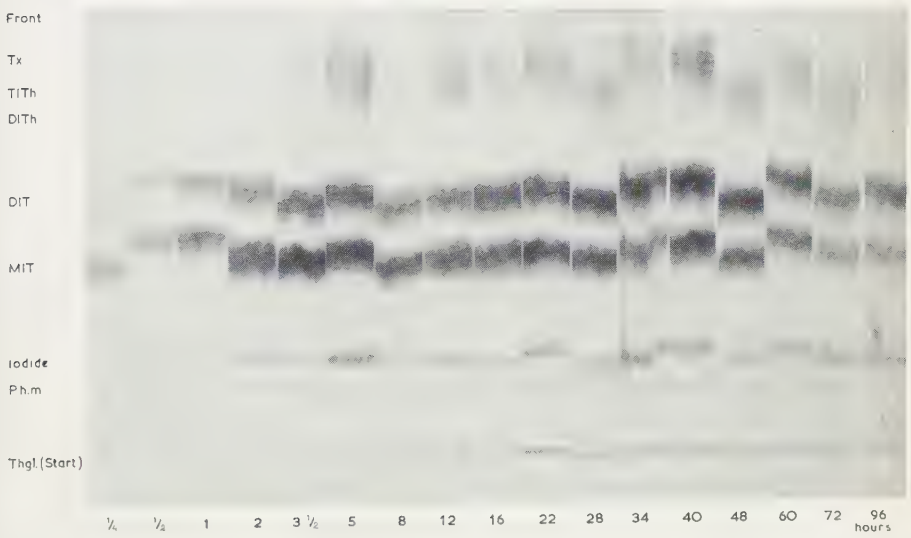

Fig. 2. Radioautograms of hydrolysed thyroid material of cockerels of Exp. II (chromatographed in but. and acet. ac.).

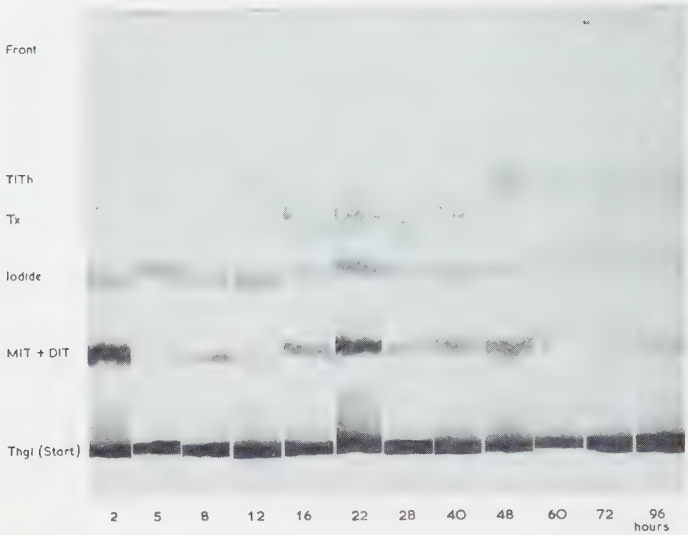

Fig. 1. Radioautograms of unhydrolysed thyroid material of cockerels of Exp. II (chromatographed in but., amm., and diox.).

Fig. 2. Radioautograms of serum of cockerels of Exp. II (chrom. in but., amm., and diox.).

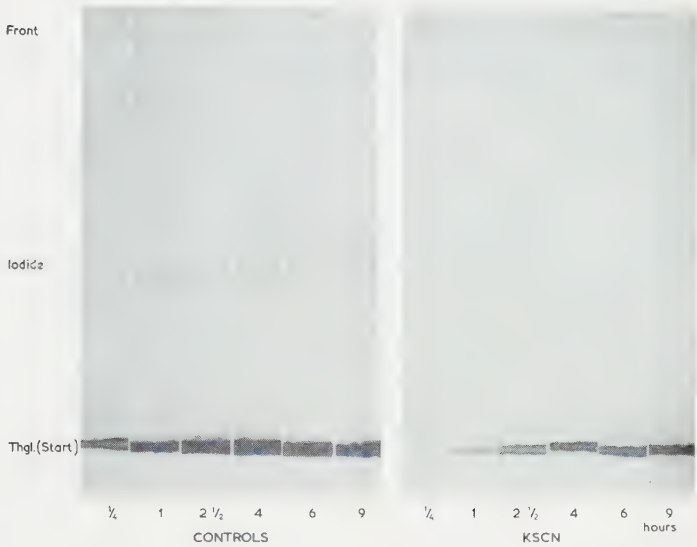

Fig. 1. Radioautograms of unhydrolysed thyroid material of normal and KSCN-treated cockerels of Exp. IV (chromatographed in but., amm., and diox.)

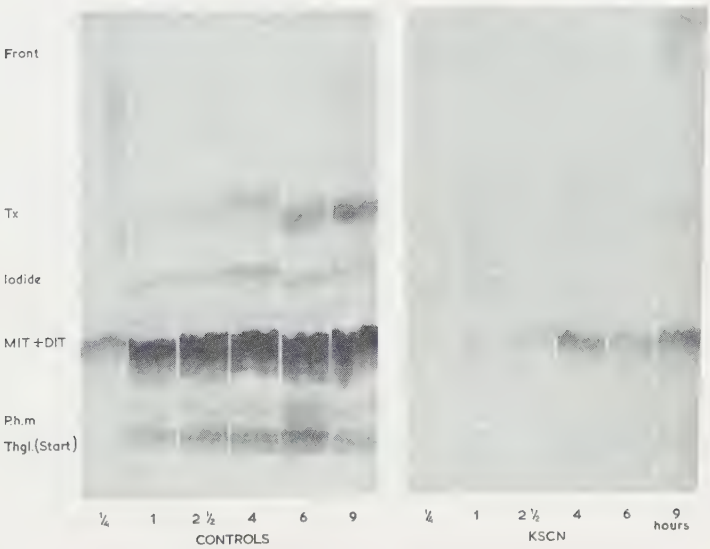

Fig. 2. Radioautograms of hydrolysed thyroid material of normal and KSCN-treated cockerels of Exp. IV (chrom. in but., amm., and diox.).

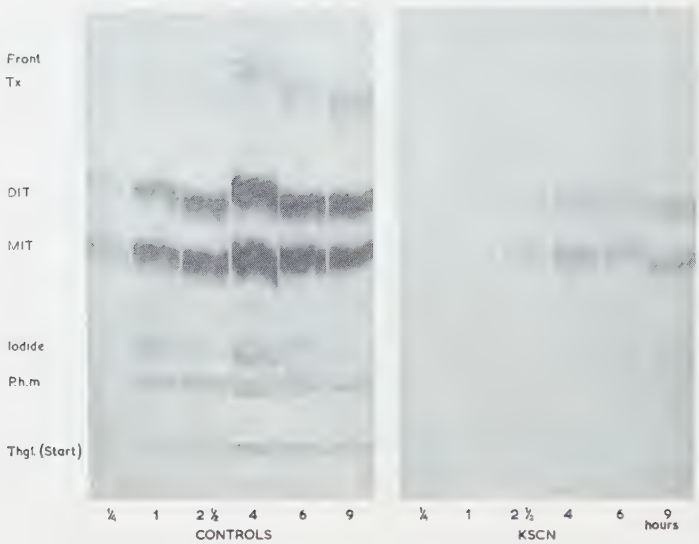

Fig. 1. Radioautograms of hydrolysed thyroid material of normal and KSCN-treated cockerels of Exp. IV (chrom. in but., and acet. ac.).

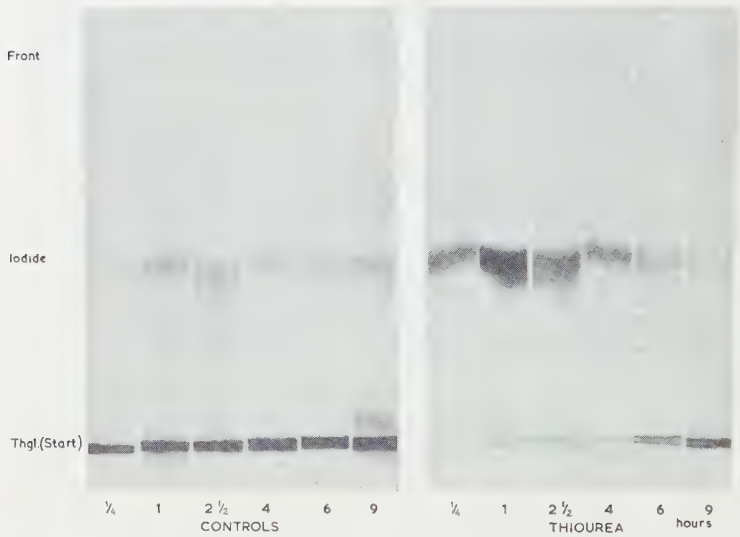

Fig. 2. Radioautograms of unhydrolysed thyroid material of normal and thiourea-treated cockerels of Exp. VI (chrom. in but., amm., and diox.).

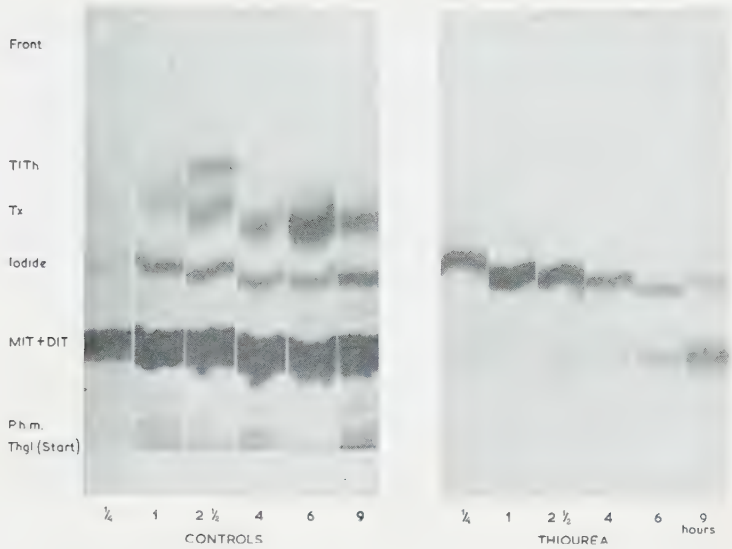

Fig. 1. Radioautograms of hydrolysed thyroid material of normal and thiourea-treated cockerels of Exp. VI (chrom. in but., amm., and diox.).

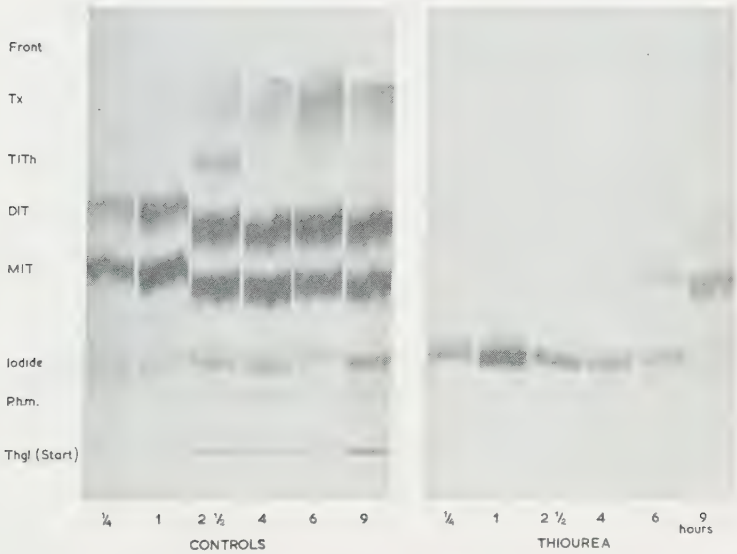

Fig. 2. Radioautograms of hydrolysed thyroid material of normal and thiourea-treated cockerels of Exp. VI (chrom. in but., and acet. ac.).

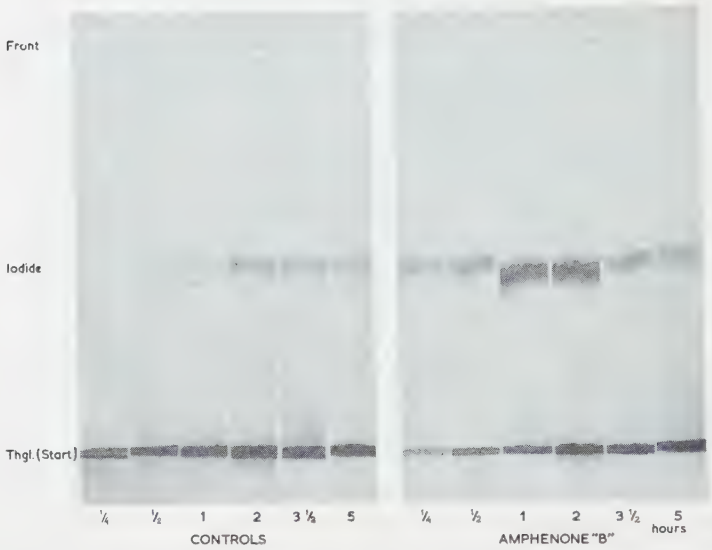

Fig. 1. Radioautograms of unhydrolysed thyroid material of normal and Amphenone 'B'-treated cockerels of Exp. VIII (chrom. in but., amm., and diox.).

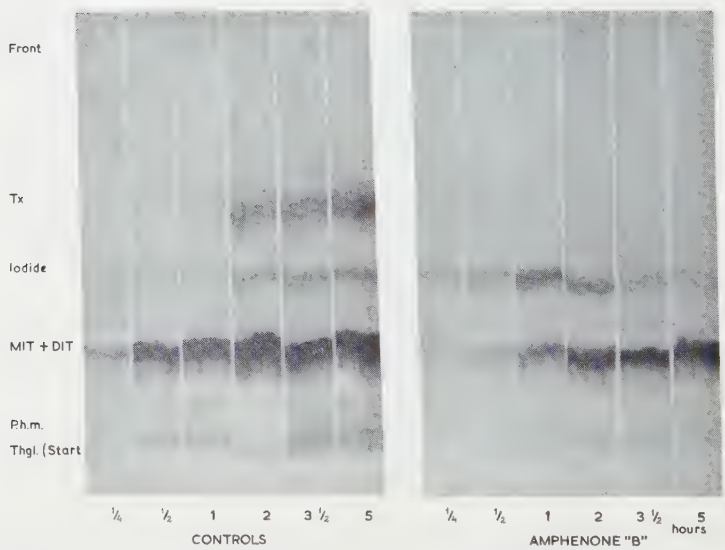

Fig. 2. Radioautograms of hydrolysed thyroid material of normal and Amphenone 'B'-treated cockerels of Exp. VIII (chrom. in but., amm., and diox.).

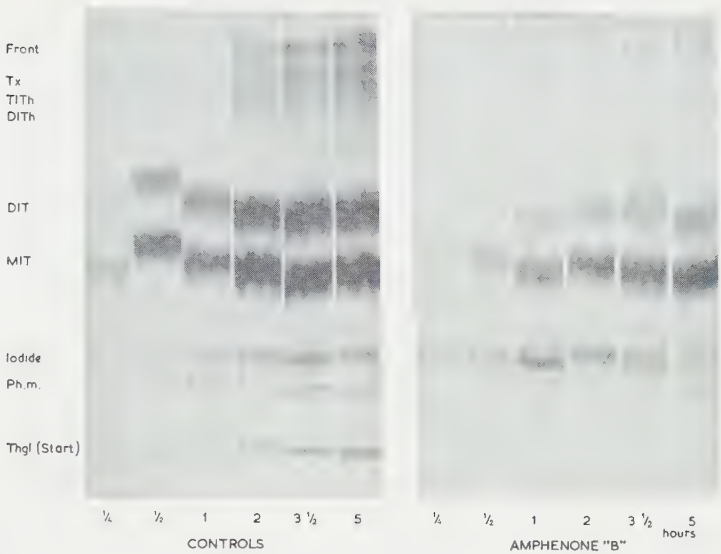

Fig. 1. Radioautograms of hydrolysed thyroid material of normal and Amphenone 'B'-treated cockerels of Exp. VIII (chrom. in but., and acet. ac.).

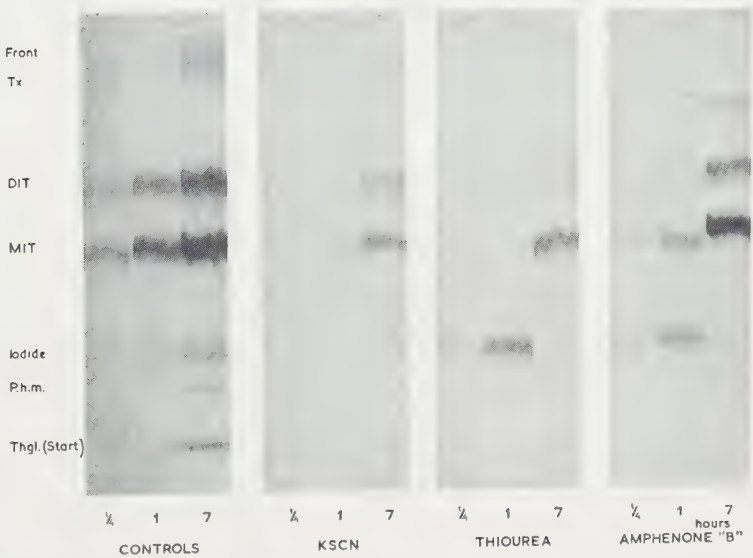

Fig. 2. Radioautograms of hydrolysed thyroid material of normal, KSCN-treated, thiourea-treated, and Amphenone 'B'-treated cockerels of Exps. III, V and VII (chrom. in but., and acet. ac.).

gland. Even the iodide which is accumulated by the thyroid cells will be flushed by KSCN (see also HALMI and STUELKE 1956, and HALMI in WOLSTENHOLME and MILLAR 1957). WIJNGAARDEN, WRIGHT and WAYS (1952) affirmed these observations, they found a number of drugs, as for example $KClO_4$, $KClO_3$, KNO_3, that share with KSCN the properties of inhibiting collection and interfering with the retention of the iodide within the thyroid gland. As WOOD and KINGSLAND (1950) failed to demonstrate the collection of KSCN in the thyroid gland, WIJNGAARDEN *et al.* are of opinion that the action of KSCN cannot be explained by a competition of KSCN with iodide. Therefore they suggest that perhaps KSCN (and compounds with the same properties) will interfere with an enzyme system which is engaged in the concentration of the iodide. By WOLFF, CHAIKOFF, TAUROG and RUBIN (1946) it was made plausible that the amount of KSCN which circulates in the blood, is a measure for the relative blocking of the iodide trap. When the amount of KSCN diminishes (which will happen early, as KSCN is excreted rather rapidly by the kidneys), gradually the effect of KSCN on the concentration of the iodide decreases. Nevertheless it has to be mentioned that LOGOTHETOPOULOS and MYANT (1956) 1 h. after an injection with thiocyanate labelled with sulphur-35 (100 γ) found the density over the colloid was 2–5 times greater than over the blood vessels and the interfollicular tissue. Therefore possibly KSCN will be concentrated in the thyroid gland, but only for a short time. RABEN (1949) has found that, after a period of blocking of the iodide concentrating mechanism of the thyroid gland, the amount of iodide which is concentrated by the thyroid gland, is increased. By these results the investigations of RAWSON, TANNHEIMER and PEACOCK (1944) can be explained. This effect seems to be caused by a direct effect on the iodide trap. It is not explainable by an increased release of thyrotropic hormone.

2. *Experiments*

It seemed to be of interest to study the action of KSCN in birds, to ascertain whether there exist some differences as compared to mammals. Also it was aimed to study the form in which the iodine was present in the thyroid glands after treatment with KSCN, to detect any qualitative and quantitative differences in the synthesis of thyroid hormones. As far as we know up to the present no study of this kind has been undertaken.

In two experiments groups of cockerels were pretreated with KSCN, 1 hour or 10 minutes before an injection with 25 μc NaI^{131}, and compared with normal cockerels.

The material for Exp. III existed of 18 cockerels, with weights varying between 750 and 1050 grams. They formed 6 groups of 3 animals each. The mean weights for the control groups were 892, 888 and 868 gms respectively, whereas the groups pretreated with KSCN had mean weights of 806, 853 and 913 gms. The mean weights of the thyroid glands for the control animals were 27.9, 31.6 and 32.2 mgs, for the pretreated animals 28.3, 28.7 and 36.7 mgs. One hour before the injection with 25 μc Na^{131}, 90 mgs KSCN were injected. The animals were killed ¼, 1 or 7 h. after the injection with radioiodide. The further treatment of the animals is described in Chapter III.

Exp. IV was carried out with 60 cockerels, their weights varying between 460 and 815 gms. They were divided into 12 groups of 5 animals each. Six groups were treated with KSCN, the other groups were controls. The mean weights for the KSCN-treated animals were 557, 536, 555, 559, 581 and 612 gms, for the untreated animals 566, 531, 548, 589, 611 and 584 gms. The mean weights of the thyroid glands amounted for the untreated animals (weight pro 5 animals) 86.5, 94.3, 97.4, 96.1, 111.4 and 116.1 mgs, for the untreated animals: 81.4, 98.0, 99.0, 132.0, 115.1 and 132.3 mgs.

In this study the experimental groups were pretreated with an injection of 90 mgs KSCN, 10 minutes before an injection with 25 μc NaI^{131}. The animals were killed ¼, 1, 2 ½, 4, 6 or 9 h. after the injection respectively.

3. *Results*

First the total radioiodine content in the experiments was determined (table XII).

TABLE XII

Total content of radioiodine in percentage of injected dose in normal and KSCN-treated animals

Time after injection of NaI^{131} in hours	Exp. III controls	Exp. III KSCN	Exp. IV controls	Exp. IV KSCN
¼	2.9	0.1	0.6	0.07
1	9.6	0.7	2.7	0.16
2 ½			4.4	0.34
4			7.7	0.77
6			6.7	0.53
7	25.4	5.4		
9			6.3	1.06

The results of these experiments are found in fig. 7. The thyroid material also was investigated by direct paperchromatography. The autoradiograms of this analysis in Exp. IV a.e reproduced in Plate III, fig. 1.

To study the partition of the iodinated amino acids, the thyroid material also was hydrolysed and analysed by paperchromatography (see Chapter III). The results of these analysis are given in table XIII. In the analysis the radioactivity in the thyronines (Tx, TITh and DITh) was taken together, as the radioactivity in TITh and DITh was very low. The autoradiograms of the paperstrips are reproduced in Plate III, fig. 2 and Plate IV, fig. 1.

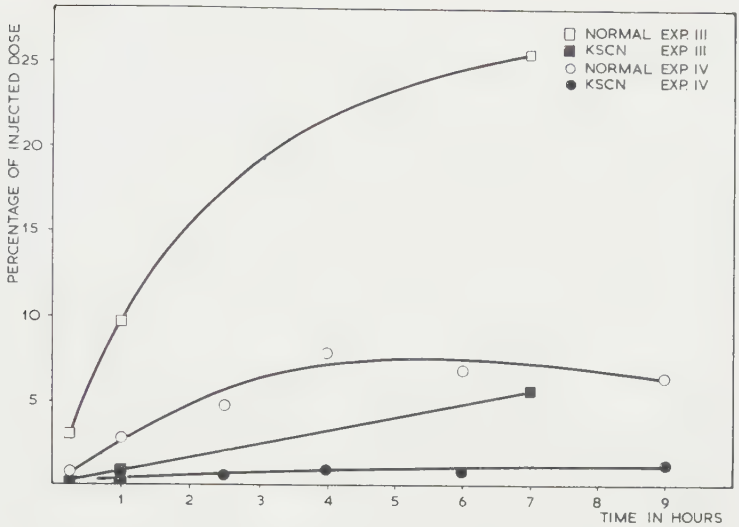

Fig. 7. Content of total radioiodine in the thyroid glands of normal and KSCN-treated cockerels, in percentage of injected dose.

TABLE XIII

Radioiodine in the different thyroid fractions, in normal and KSCN-treated animals. Left: radioiodine in percentage of injected dose (total). Right: radioiodine in percentage of concentrated dose (relative)

	Time in hours after inj. of I^{131}	Iodide total	Iodide relative	MIT total	MIT relative	DIT total	DIT relative	Tx total	Tx relative
controls	¼	0.19	6.5	1.85	63.8	0.82	28.4	0.04	1.3
	1	0.53	5.5	5.26	54.8	3.51	36.6	0.30	3.1
	7	2.23	8.8	11.99	47.2	7.52	29.6	3.66	14.4
Exp. I									
KSCN	¼	—	—	0.09	90.0	0.01	10.0	—	—
	1	0.02	3.1	0.54	76.8	0.14	20.1	—	—
	7	0.63	11.6	3.01	55.8	1.52	28.2	0.24	4.4
controls	¼	0.04	6.7	0.36	60.0	0.20	33.3	—	—
	1	0.18	6.7	1.46	54.1	0.97	35.9	0.09	3.3
	2 ½	0.24	5.5	2.33	52.9	1.57	35.7	0.26	5.9
	4	0.46	6.0	3.68	47.8	2.88	37.4	0.68	8.8
	6	0.29	4.3	3.16	48.7	2.48	35.5	0.77	11.5
	9	0.30	4.8	2.54	40.3	2.59	40.9	0.88	14.0
Exp. II									
KSCN	¼	—	—	+	80?	+	20?	—	—
	1	+	+	0.11	68.7	0.05	32.3	—	—
	2 ½	0.01	3.0	0.21	61.7	0.12	35.3	—	—
	4	0.03	4.0	0.42	54.5	0.32	41.5	—	—
	6	0.04	7.5	0.32	60.3	0.14	26.5	0.03	5.7
	9	0.05	4.7	0.51	48.2	0.40	37.7	0.10	9.4

4. *Discussion*

From fig. 7 it is clear that the thyroid gland, treated with KSCN, has a lower iodine content as compared to controls. The content of radioiodine in the thyroid gland increases with time, but this increase is much less than in untreated animals. The smaller content of iodine obviously is caused by the fact that after treatment with KSCN less iodide is concentrated within the gland.

Plate III, fig. 1 shows that practically no radioiodide is demonstrated autoradiographically (see also Plate III, fig. 2 and Plate IV, fig. 1). From table XIII it is clear that in chromatographical analysis, followed by counting with a β-counter, no iodide at all is demonstrable in the first hours after the treatment. So we may conclude that in birds, in accordance with the results in mammals of WOLFF, CHAIKOFF, TAUROG and RUBIN (1946), VANDERLAAN and VANDERLAAN (1947) and WIJNGAARDEN, WRIGHT and WAYS (1952), the treatment with KSCN blocks the iodide trap. About the mode of action of KSCN the experiments are not decisive. As was mentioned before, on this point opinions differ.

Also, however, some synthesis of hormones happens even in the KSCN-treated animals, as shown by the presence of I^{131}-thyroglobulin in unhydrolysed material (Plate III, fig. 1), and the presence of iodinated amino acids after hydrolysis (Plate III, fig. 2 and Plate IV, fig. 1). As MIT, DIT, and after some time also Tx are demonstrated, the differences obviously are not qualitative (compare also fig. 12).

To gain some better understanding of the processes in normal animals, it is necessary to go further into the differences existing between normal and KSCN-treated animals. This is possible by calculating MIT/DIT and DIT/Tx in the two experiments (table XIV).

TABLE XIV

Quotients MIT/DIT and DIT/Tx in Exps. III and IV

	Time in hours after injection	MIT/DIT controls	MIT/DIT KSCN	DIT/Tx controls	DIT/Tx KSCN
Exp. III	¼	2.2	9.0	21.8	∞
	1	1.5	3.8	11.9	∞
	7	1.6	2.0	2.1	6.4
Exp. IV	¼	1.8	4	∞	∞
	1	1.5	2.1	10.7	∞
	2 ½	1.5	1.7	6.0	∞
	4	1.3	1.3	4.2	∞
	6	1.3	2.3	3.2	4.6
	9	1.0	1.3	2.9	4.0

In the treated animals evidently identical changes in the quotient MIT/DIT happen as in the normal animals studied in Chapter IV. In the KSCN-treated animals MIT/DIT obviously is higher than in controls, but here also a gradual decrease in the values can be noted.

In Chapter IV it was suggested that the initial decrease of the value of MIT/DIT is caused by the fact that shortly after the injection of radioiodide DIT only is formed by iodination of nonradioactive MIT with radioiodine, whereas afterwards DIT also comes into existence by iodination of radioactive MIT with iodine[127]. According as the second process gains in importance, DIT is formed at a higher rate as compared to MIT.

Apparently the same holds for the KSCN-treated animals, though MIT/DIT decreases more slowly as compared to untreated animals, as only after some hours the quotient in the KSCN-treated animals attains the value of the untreated animals after $^1/_4$ h. This implies that in the treated animals DIT is formed at a slower rate. Probably this is caused by the fact that the amount of iodine atoms to iodinate the amino acids in the experimental animals is lower as compared to controls. The data on the quotient DIT/Tx, summarized in table XIV, confirm the impression that, under the influence of a treatment with KSCN, the synthesis of thyroid hormones will happen at a lower rate.

The foregoing remarks suggest that KSCN acts indirectly on the iodination and condensation processes within the thyroid gland. No qualitative difference exists between treated and untreated animals (see Chapter VIII). The fact that after some time MIT/DIT in treated and untreated animals shows a great resemblance has to be mentioned. It is evident that in Exp. III the difference in MIT/DIT for all groups is greater than in Exp. IV. Thus in Exp. III there has been a greater response to the KSCN than in Exp. IV. This may be caused by the fact that the thyroid glands of the control-animals in Exp. III have accumulated more radioiodine as compared to those of Exp. IV.

Nevertheless the action of KSCN as seen in MIT/DIT in both experiments is of the same kind. If there were any direct action of KSCN on the iodination and condensation processes, which escaped attention in Exp. III due to the 1-hour pretreatment, some irregularity in the values of MIT/DIT in Exp. IV could be expected, for in Exp. IV $^1/_4$ h. after the injection with NaI^{131} (25 minutes after the treatment with KSCN) the response of the thyroid gland was not yet maximal. This may be seen by the quotient proteinbound I^{131} in KSCN-treated animals/proteinbound I^{131} in untreated animals (Table XV).

If any special action on the iodination processes existed, in Exp. IV some relation between blocking percentage and MIT/DIT would be

TABLE XV

The percentage of blocking by KSCN in Exps. III and IV.
(Proteinbound I^{131} in KSCN treated animals in percentage of that of control animals)

Time after injection of NaI^{131} in hours	Relative blocking by KSCN in Exp. III	Exp. IV
¼	3.7	12.5
1	7.5	6.3
2 ½		7.9
4		10.2
6		7.7
7	20.6	
9		16.8

found. As this is not the case, no differences at all are demonstrable between the two experiments, indicating that to some extent changes in MIT/DIT are independent with respect to the time which passes between the injection of KSCN and the killing of the animals, when the effect of KSCN has not vanished at all. So it is concluded that, when animals are pretreated with KSCN, only the number of iodine atoms, which is capable of iodination of tyrosine-radicals in the thyroglobulin, is the limiting factor in the processes which are involved in the synthesis of thyroid hormones. Probably by gradual diminution of the blocking action, after some time the values of MIT/DIT in treated and untreated animals resemble each other. This effect however would not occur in Exp. IV if any influence upon the iodination existed.

The experiments, performed with birds, are in keeping with the results obtained by several authors in mammals. KSCN inhibits the iodide concentrating mechanism. From the experiments it is concluded that KSCN merely indirectly affects the iodination and condensation processes involved in the synthesis of the thyroid hormones. These processes slow down, but qualitatively no differences as compared to normal animals are detected (see Chapter VIII).

VI. THE SYNTHESIS OF THYROID HORMONES IN THIOUREA-TREATED COCKERELS

1. *Literature*

The number of papers on the action of thiourea and its derivatives is very extensive. We will review only some of the investigations, which show clearly the effects of the thiourea-derivatives. The literature on this subject is reviewed by Astwood (1949), Pitt-Rivers (1950), Albert (1952), and Vanderlaan and Storrie (1955). Mixner, Reinecke and Turner (1944) demonstrated a goitrogenic activity of

thiouracil in one day old chickens. LARSON, KEATING, PEACOCK and RAWSON (1945), also using chickens as experimental animals, showed that treatment with thiouracil strongly decreased the accumulation of radioactive iodine in the thyroid gland. They supposed this effect was due to a blocking action of the iodide concentration mechanism, which afterwards was proved to be a false theory. FRANKLIN, LERNER and CHAIKOFF (1944) also found in rats a decrease in the content of radioiodine after treatment with thiourea or thiouracil. This study was affirmed by ASTWOOD and BISSELL (1946). These authors found that the synthesis of hormones occurred to a lesser extent, though their experiments failed to elucidate the factors causing this decrease. This was studied by VANDERLAAN and BISSELL (1946) in rats. They were able to show that, after treatment with propylthiouracil, the concentration of iodide within the thyroid gland occurred as usually. The maximum uptake was found to be about 30 min. after the administration of the iodide. However, after 5 to 6 h. half of the concentrated iodine has been released by the thyroid gland, whereas after about 24 h. the iodine content of the gland is the same as before. Thus the concentration of the iodide occurs in a normal way, even more iodide is concentrated in the treated than in untreated animals, but the thyroid gland is not able to retain this iodide, as the iodine cannot be incorporated in the thyroglobulin. On these points all authors agree. With respect to the mode of action of thiourea and its derivatives, some diversity of opinion can be found. PITT-RIVERS (1948, 1950) claims a direct action of thiourea and its derivatives, which would be explained by mere chemical principles. She points to the fact that in vitro a rapid reduction of iodine to iodide by thiourea can be demonstrated: therefore also in vivo possibly no iodine can be formed. So in vitro iodination of tyrosine-radicals is inhibited by thiourea. PITT-RIVERS (1948) mentions that in vitro also the condensation of acetyldiiodotyrosine to acetylthyroxine is inhibited by thiourea.

TAUROG, CHAIKOFF and FRANKLIN (1945) demonstrated in vitro a relation of the inhibition of the conversion of DIT to Tx by 40 antithyroid drugs to their oxidizability. However no direct grounds for this conception by in vivo experiments has been found. FAWCETT and KIRKWOOD (1953) proposed a modification of the conception of PITT-RIVERS. They suggest that the action of antithyroid drugs of the kind of thiourea is based upon a direct chemical binding of the agens with elementary iodine. There are some objections to this theory, for example in paper-chromatographical analysis the iodine was detected as iodide (see VANDERLAAN and STORRIE 1955).

On the other hand some investigators claim an indirect action of thiourea and its derivatives, based upon the inhibition of an enzyme

system governing the oxidation of iodide to iodine. We may conclude that, due to lack of experimental data, the opinions about the mode of action of thiourea and its derivatives are divergent.

Though we mentioned that KSCN is not, or to a lesser extent (WOOD and KINGSLAND 1950; LOGOTHETOPOULOS and MYANT 1956) concentrated within the thyroid gland, there exist several investigations which point to a concentration of thiourea. BEZEM, BRUNNEKREEFT, ERNSTING, LEVER and NAUTA (1949) detected in cocks, 24 h. after administration of radioactive methylthiouracil, important amounts of radioactive S within the thyroid gland as opposed to most other tissues. They were not able however to determine which quantity was in the form of MTU, or in the form of katabolic products of MTU. Also VAN ASPEREN (1951), employing a colorometric determination of methylthiouracil, concluded that the amount of MTU in the thyroid gland was higher, as compared to other tissues.

It seemed of interest to study with the techniques described the processes involved in the synthesis of the thyroid hormones, in birds, after administration of thiourea.

2. *Experiments*

To study the effect of thiourea upon the thyroid processes, two experiments were carried out. In Exp. V 1 h. before an injection with radioiodide, groups of cockerels were pretreated with an injection of 37.5 mg thiourea. In Exp. VI the thiourea was administered 10 min. before the injection with radioiodide. In both experiments groups of cockerels were killed at different times after the injection with radioiodide.

The material of Exp. V existed of 18, 8-9 weeks old, cockerels. They were killed in 6 groups of 3 animals each. The controls of this experiment were the same as in Exp. III (Chapter V). The experimental groups had mean weights of 783, 783 and 842 gms. The mean thyroid weights were 32.3, 28.7 and 37.6 mgs. The animals were pretreated, 1 hour before the injection with NaI^{131}, with 37.5 mg thiourea (in 1 ml of a 0.9% NaCl-solution). They were killed at ¼, 1 or 7 h. respectively after the injection of NaI^{131}.

Exp. VI was carried out with 60, 7½ weeks old, cockerels. Their weights varied between 440 and 610 gms. They were divided into 12 groups (of 5 animals each), 6 groups formed the controls. The mean weights of the controls were 522, 528, 540, 539, 542 and 519 gms, of the thiourea-treated animals 524, 527, 551, 554, 563 and 532 gms. The total weights of the thyroid glands of one group amounted for the controls: 100.5, 113.3, 121.8, 108.2, 121.4 and 121.5 mgs, for the treated animals: 123.7, 122.8, 110.8, 120.0, 150.4 and 135.4 mgs. The experimental animals were pretreated, 10 min. before the injection with radioiodide, with 37.5 mgs thiourea in 1 ml of a 0.9% NaCl-solution.

Treated and untreated animals were killed ¼, 1, 2½, 4, 6 or 9 h. after the injection with radioiodide. The technique which was employed is communicated in Chapter III.

3. *Results*

First in the two experiments the total radioiodine content of the thyroid gland was determined. The results are shown in table XVI and fig. 8.

TABLE XVI

Total content of radioiodine in percentage of injected dose in normal and thiourea-treated animals

Time after injection of NaI^{131} in hours	Exp. V controls	Exp. V thiourea	Exp. VI controls	Exp. VI thiourea
¼	2.9	1.0	1.9	1.2
1	9.6	3.5	8.3	3.2
2 ½			16.6	1.6
4			17.2	1.3
6			16.6	1.1
7	25.4	4.0		
9			19.4	1.4

To study the amount of inorganic and proteinbound radioiodine, the thyroid material was analysed by paperchromatography. The results of the autoradiograms of the paper strips of Exp. VI are reproduced in Plate IV, fig. 2.

Moreover the material was hydrolysed to study the partition of the proteinbound I^{131} among the different fractions. The results of autoradiograms of Exp. VI are reproduced in Plate V, figs. 1 and 2. The results of the hydrolysed material in Exps. V and VI are given in table XVII.

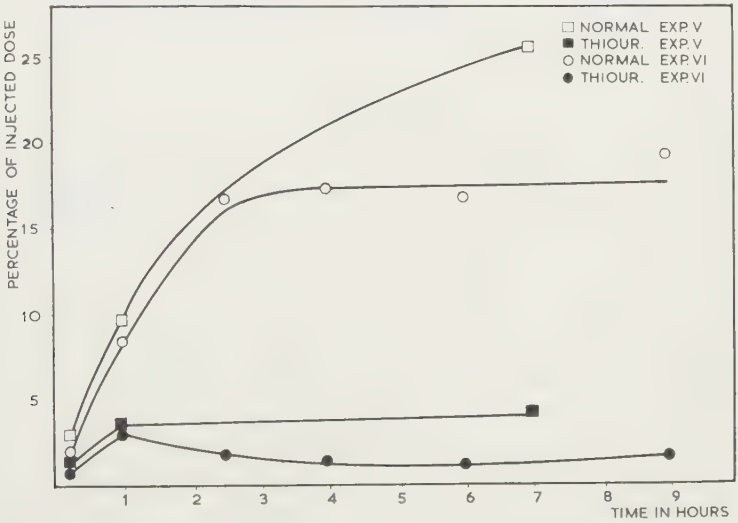

Fig. 8. Content of total radioiodine in the thyroid glands of normal and thiourea-treated codkerels, in percentage of injected dose.

TABLE XVII

Radioiodine in the different fractions, in normal and thiourea-treated animals. Left: radioiodine in percentage of injected dose (total). Right: radioiodine in percentage of concentrated dose (relative)

	Time after the inj. of NaI^{131} in hours	Iodide total	Iodide relative	MIT total	MIT relative	DIT total	DIT relative	Tx total	Tx relative	TITh total	TITh relati
	¼	0.19	6.5	1.85	63.8	0.82	28.4	0.04	1.3		
controls	1	0.53	5.5	5.26	54.8	3.51	36.6	0.30	3.1		
	7	2.23	8.8	11.99	47.2	7.52	29.6	3.66	14.4		
Exp. V											
	¼	1.0	100	—	—	—	—	—	—		
thiourea	1	3.36	96.0	0.14	4.0	—	—	—	—		
	7	0.17	4.2	3.25	81.3	0.53	13.2	0.05	1.3		
	¼	0.06	3.2	1.22	64.2	0.62	32.6	—	—	—	—
	1	0.64	7.8	4.27	52.6	2.86	33.2	0.53	6.4	—	—
controls	2 ½	1.83	11.0	5.95	35.9	5.32	32.0	2.01	12.1	1.49	9.0
	4	1.38	8.0	7.53	43.8	6.14	35.7	1.93	11.2	0.22	1.3
	6	0.99	6.0	5.87	35.4	5.73	34.4	3.58	21.6	0.43	2.6
	9	2.75	14.2	7.51	38.7	6.09	31.4	2.64	13.6	0.41	2.1
Exp. VI											
	¼	1.17	96.4	0.02	2.4	0.01	1.2	—	—	—	—
	1	3.13	97.1	0.08	2.1	0.03	0.8	—	—	—	—
thiourea	2 ½	1.47	91.6	0.08	5.4	0.05	3.0	—	—	—	—
	4	1.16	84.0	0.14	9.2	0.05	3.4	0.05	3.4	—	—
	6	0.69	62.0	0.27	25.0	0.10	9.0	0.04	4.0	—	—
	9	0.30	20.8	0.77	56.3	0.20	13.9	0.13	9.0	—	—

4. *Discussion*

As is shown clearly by the experiments thiourea is a powerful inhibitor of the accumulation of iodide in the thyroid gland (fig. 8). Initially the uptake is not strongly inhibited (see table 16, uptake ¼ and 1 h. after the injection), but, especially after some time, a low amount of radioiodine could be detected in the treated animals, as compared to the untreated animals. In Exp. VI the radioiodine content in the glands of the thiourea-treated animals even diminished. In Exp. V this decrease could not be observed, as the number of observations was too small.

What happens in the thyroid gland under the influence of treatment with thiourea, is clearly demonstrated bij Plate IV fig. 2. which shows the autoradiograms of the chromatograms of unhydrolysed thyroid material. In the untreated animals the radioiodine is almost exclusively found in the thyroglobulin. On the contrary in the thiourea-treated animals, especially during the first hours after the injection with radio-

iodide, the radioiodine is mainly in the form of iodide. The same is shown by the radioautograms of the hydrolysed material (Plate V, figs. 1 and 2). From the abovementioned results it is concluded that the thyroid gland is able to concentrate iodide. This process apparently is autonomous with respect to the binding of the iodine in the thyroid protein. The amount of iodide in the thiourea-treated animals is even higher as compared to normal animals (table XVII). As no iodination of the thyroglobulin occurs (or only to a very small extent), and the radioiodine is demonstrated in the form of iodide, most probably the oxidation of iodide to elementary iodine (perhaps an enzymatic process) which normally occurs, is inhibited. As a result iodination of amino acids also is impossible. So the processes in birds after treatment with thiourea, are comparable with those described for mammals (see f.i. VANDERLAAN and BISSELL 1946).

Table XVII, which shows the relative amount of radioiodine in the different processes, clearly demonstrates that only to the end of the experiments the amounts of radioiodine in amino acids are notable. Though the amounts of radioiodine in the thiourea-treated animals are very low, which causes a rather inaccurate value of the quotient MIT/DIT, it may be interesting to discuss the values of MIT/DIT and DIT/Tx reproduced in table XVIII.

TABLE XVIII

Quotients MIT/DIT and DIT/Tx in Exp. V and VI

	Time in hours after injection	MIT/DIT control	MIT/DIT thiourea	DIT/Tx[1] control	DIT/Tx[1] thiourea
Exp. V	¼	2.2	—	21.8	—
	1	1.5	∞	11.9	—
	7	1.6	6.1	2.1	10.2
Exp. VI	¼	2.0	2.0	∞	—
	1	1.5	2.7	5.4	—
	2 ½	1.1	1.6	2.7	—
	4	1.2	2.8	3.2	1.0
	6	1.0	2.7	1.6	2.2
	9	1.2	3.9	2.3	1.5

[1] In calculating the value of DIT/Tx in the untreated animals, the values of TITh are included. In the treated animals no TITh was demonstrable.

In both experiments MIT/DIT is higher in the treated animals as compared to the untreated animals. From the experiments with KSCN (Chapter V) it was concluded that the rise in the value of MIT/DIT probably was caused by the presence of smaller amounts of iodine to iodinate the amino acids. Possibly the phenomena, observed in the thiourea-treated animals, are of the same kind. The relative unrealibi-

lity of the first values in Exp. VI, due to the minute amounts of radioiodine detected, was mentioned before. Therefore it is impossible to draw any further conclusion here. We will return to this point in Chapter VIII.

The data about Tx permit no conclusions. It is impossible to observe in the experiments any inhibition of the condensation reaction DIT → Tx, which was shown by PITT-RIVERS (1948) to occur in vitro. The occurrence of Tx in our experiments easily can be explained by a decline in inhibition. As for the one high value of radioiodine in 3 : 5 : 3′-TITh, demonstrated in the untreated animals (see Plate V, figs. 1 and 2), this is rather remarkable. Most probably this is due to some irregularity in the thyroid gland of one cock of this group. It is impossible to draw any further conclusions.

The rate of inhibition can be shown by comparing the amounts of protein bound radioiodine in the thiourea-treated animals, as compared to the control animals (table XIX).

TABLE XIX

The percentage of blocking by thiourea in Exps. V and VI. (Proteinbound I^{131} in thiourea-treated animals in percentage of that of control animals)

Time after injection of NaI^{131} in hours	Relative blocking by thiourea in Exp. V	Exp. VI
¼	0	2.2
1	1.7	1.2
2 ½		1.0
4		1.3
6		2.6
7	23.7	
9		6.8

Evidently in Exp. V a maximal effect of thiourea was obtained earlier than in Exp. VI. This however probably is related to the time of administration of the thiourea, which in Exp. V was earlier than in Exp. VI. The inhibition in Exp. VI lasted for a longer time, as compared to Exp. V, though in Exp. V the number of observations was smaller. Most probably this is related to the weights of the experimental animals and the activity of the thyroid glands (see also Chapter VIII).

Obviously a gradual decrease in the inhibition occurs. The amount of protein-bound I^{131} increases with time, whereas the amount of I^{131} in inorganic form decreases. In the first period following the injection the decrease of inorganic iodine beats the increase of protein-bound I^{131}. So radioiodine is released in inorganic form from the gland: it cannot be retained. Not until 4 h. after the injection (in Exp. VI) the

greater part of the iodide probably is used for the iodination of amino acids.

Thus our results agree with those of VANDERLAAN and BISSELL (1946). These authors, however, employed very high quantities of iodine. Our experiments do not decide exactly between the two conceptions about the action of thiourea mentioned before. It remains unclear whether this must be explained by a direct chemical reaction (PITT-RIVERS 1948, 1950) or by an indirect inhibition of an enzyme system, for example the peroxidase system (ROSENBERG 1953). However the theory of FAWCETT and KIRKWOOD (1953) is not supported by our observations, as by chromatographical analysis the inorganic iodine was shown to be iodide.

VII. THE SYNTHESIS OF THYROID HORMONES IN AMPHENONE "B"-TREATED COCKERELS

1. *Literature*

Amphenone "B" (1-2-*bis*-(p-aminophenyl)-2-methylpropanone-l-dihydrochloride), synthesized by ALLEN and CORWIN (1950) is proved to affect different organs. As shown by HERTZ, ALLEN, TULLNER and WESTFALL (1952), beside having a general anaesthetic effect, it influences the epithelium of the uterus, the adrenal and the thyroid gland. As to the lastmentioned organ, HERTZ, ALLEN and TULLNER (1950) demonstrated in rats, treated with Amphenone "B", hyperplasia of the thyroid gland. The follicles were almost free from colloid, whereas the epithelium cells were of the tall cylindrical type. The action of Amphenone "B" is, as observed by HERTZ, TULLNER and ALLEN (1951), opposed by thyroxine. HEMING, HOLTKAMP, KERWIN, MANSOR and DACANAY (1952) demonstrated a lack of function of the treated glands, as they could observe a diminished oxygen consumption. HOGNESS, WILLIAMS and LANCE (1952) showed a decrease in the accumulation of radioiodine within the thyroid gland (the content was 16% of the amount concentrated by normal animals), whereas HOGNESS, LEE and WILLIAMS (1953) found, when Amphenone "B" was administered intravenously, a decrease of iodine accumulation up to 90%. They tried, by comparing the effect of Amphenone "B" with that of propylthiouracil, to investigate whether it affected the iodide concentration or the hormone release. Neither PTU nor Amphenone "B" showed any influence on the release of the hormones. In view of the analogy in the effects of both antithyroid drugs the authors argue for an analogy in the mode of action.

ADAMS and BENNETT (1953), treating chicken embryos with Am-

phenone "B", showed similar histological effects in the thyroid glands. The thyroid glands were hypertrophic and hyperplastic.

LEVER and VAN OVERBEEKE (1956), with the aid of several histological techniques, studied in cockerels the potency of Amphenone "B" as an antithyroid drug, by comparing the histological response of different doses with that of 50 mgs methylthiouracil. They affirmed the results of ADAMS and BENNETT and concluded that in the technique used (the antithyroid drugs were orally administered in tragacanth pills) 110 mgs Amphenone "B" were equivalent to 50 mgs MTU.

From the literature cited above it can be seen that no exact data are available about the mode of action of Amphenone "B". Though HERTZ, PITTMAN and GRAFF (1956), treating man with Amphenone "B", observed no KSCN-effect (on the iodide concentrating mechanism) of this compound, it seemed of interest to study by paperchromatographical analysis the effects of Amphenone "B" on the accumulation of radioiodine in the thyroid gland of cockerels.

2. *Experiments*

The effect of Amphenone "B" on the accumulation of radioiodine and the partition of the radioiodine among the different fractions was studied in two experiments (Exps. VII and VIII). In Exp. VII the animals were pretreated with Amphenone "B" 1 h. before an injection with radioiodide, in Exp. VIII 10 min. before this injection the animals were pretreated.

Exp. VII was carried out with 18 cockerels in the age of about 8–9 weeks. In this experiment, as in Exps. III and V, the same control animals were used. The mean weights of the groups pretreated with Amphenone "B" were 825, 818 and 825 gms. The mean thyroid weights were determined as to be 36.5, 35.1 and 31.2 mgs.

The experimental animals were treated with an injection of 110 mgs Amphenone "B" in 1 ml of a 0.9% NaCl-solution, 1 hour before an injection with 25 μc NaI^{131}.

The animals were killed ¼, 1 or 7 h. respectively after the injection with radioiodide.

Exp. VIII was carried out with 60 cockerels. The weights varied between 545 and 815 gms. They were divided into 12 groups of 5 animals each, 6 groups were treated with Amphenone "B". The mean weights of the animals of the control-groups were 687, 660, 655, 668, 663 and 665 gms, those of the treated groups: 681, 659, 670, 665, 662 and 662 gms. The total thyroid weights were for the control groups 109.7, 109.6, 110.4, 106.7, 102.9 and 99.9 mgs, for the Amphenone "B"-treated groups 135.8, 95.0, 111.1, 100.7, 109.1 and 83.2 mgs.

The Amphenone "B" (110 mg in 1 ml of a 0.9% NaCl solution) was injected 10 min. before they received an injection with 25 μc NaI^{131}.

The groups of treated and untreated animals were killed ¼, ½, 1, 2, 3½ or 5 h. after the injection with radioiodide. For the techniques employed see Chapter III.

3. *Results*

First the content of radioiodine in the thyroid gland at different times after the injection with radioiodide was determined. Amphenone "B" apparently diminishes the radioiodine content (see table xx).

TABLE XX

Total content of radioiodine in percentage of injected dose in normal and Amphenone "B"-treated animals

Time after injection NaI^{131} in hours	Exp. VII controls	Exp. VII Amph. "B"	Exp. VIII controls	Exp. VIII Amph. "B"
¼	2.9	1.0	0.8	0.4
½	9.6	3.3	3.2	0.9
1			3.9	2.4
2			12.4	3.6
3 ½			12.4	3.1
5			12.6	3.7
7	25.4	11.8		

These results are shown in fig. 9.

The thyroid material also was investigated by paper chromatography. The autoradiograms of this analysis in Exp. VIII are reproduced in plate VI fig. 1. Moreover, to study the partition of the radioiodine among the different fractions, the thyroid material was hydrolysed and analysed by paper chromatography (table XXI, Plate VI fig. 2, Plate VII fig. 1).

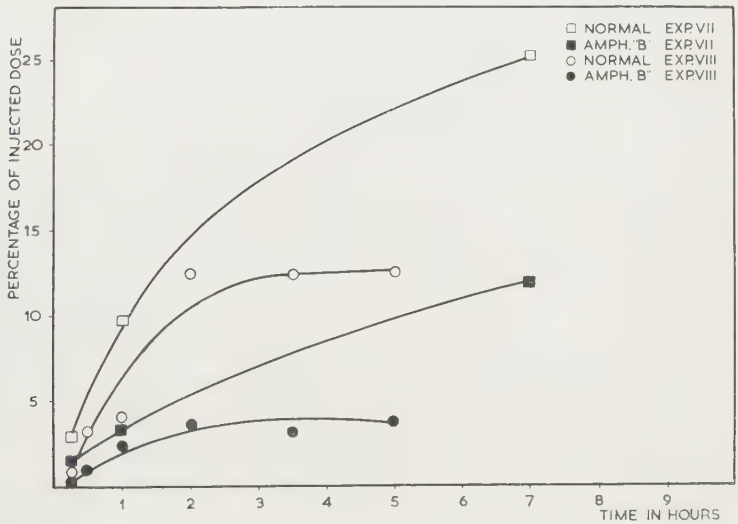

Fig. 9. Content of total radioiodine in the thyroid glands of normal and Amphenone "B"-treated cockerels, in percentage of injected dose

TABLE XXI

Radioiodine in the different thyroid fractions, in normal and Amphenone "B"-treated animals. Left: radioiodine in percentage of injected dose (total). Right: radioiodine in percentage of concentrated dose (relative)

	Time after inj. of NaI^{131} in hours	Iodide total	Iodide relative	MIT total	MIT relative	DIT total	DIT relative	Tx total	Tx rela
	¼	0.19	6.5	1.85	63.8	0.82	28.4	0.04	1
controls	1	0.53	5.5	5.26	54.8	3.51	36.6	0.30	3
	7	2.23	8.8	11.99	47.2	7.52	29.6	3.66	14
Exp. VII									
	¼	0.60	60.4	0.35	35.0	0.05	4.6	—	—
Amphenone "B"	1	1.62	48.8	1.45	44.0	0.21	6.5	0.02	0
	7	0.57	4.8	7.90	67.0	2.89	24.5	0.44	3
	¼	0.05	7.0	0.55	70.5	0.20	22.5	—	—
	½	0.25	7.7	2.02	64.2	0.80	24.2	0.13	3
controls	1	0.24	6.0	2.05	52.7	1.44	36.8	0.17	4
	2	0.93	7.5	5.99	48.6	3.69	29.5	1.79	14
	3 ½	1.13	9.1	5.40	44.0	3.76	29.9	2.11	17
	5	0.97	7.7	5.08	40.2	3.68	28.7	2.97	23
Exp. VIII									
	¼	0.27	67.0	0.11	28.0	0.02	5.0	—	—
	½	0.51	56.7	0.31	35.0	0.08	8.3	—	—
Amphenone "B"	1	1.15	47.5	0.98	41.1	0.27	11.4	—	—
	2	0.84	23.4	2.04	56.3	0.64	18.1	0.08	2
	3 ½	0.68	21.6	1.76	57.4	0.53	17.0	0.13	4
	5	0.34	9.1	2.50	67.5	0.73	19.8	0.13	3

4. *Discussion*

As observed by other authors, Amphenone "B" acts as an antithyroid drug. This is clearly demonstrated by table XX and fig. 9. The radioiodine content in the thyroid glands of the treated animals in both experiments is remarkably lower as compared to the control animals.

From table XXI and the autoradiograms of the unhydrolysed thyroid material (Plate VI, fig. 1) it may be concluded that the relative amounts of inorganic and protein-bound radioiodine in the treated animals with respect to the untreated animals, have changed. Proportionally more radioiodine is demonstrated in the form of iodide. These data may be summarized as follows: the thyroid gland, treated with Amphenone "B", is capable of concentrating radioiodine, it is unable however to incorporate all this iodine in the thyroglobulin. Amphenone "B" has an identical effect on the processes involved in the production of thyroid hormones as thiourea, i.e. it inhibits the conversion of iodide to iodine (VLIJM, 1956). The experiments indicate an autonomy of the iodide concentrating mechanism, as was concluded from the experi-

ments with thiourea. Nevertheless there exist rather high amounts of radioiodine in protein bound form (Plate VI, fig. 2, Plate VII, fig. 1). It may be interesting to study once more the ratios of the radioiodine in the different fractions.

These ratios may be studied by calculating the quotients MIT/DIT and DIT/Tx in the two experiments (table XXII).

TABLE XXII

Quotients MIT/DIT and DIT/Tx in Exps. VII and VIII

	Time in hours after injection	MIT/DIT control	MIT/DIT Amph. "B"	DIT/Tx control	DIT/Tx Amph. "B"
Exp. VII	½	2	7.5	21.8	∞
	1	1.5	7	11.9	9.3
	7	1.6	3	2.1	6.6
Exp. VIII	¼	2.7	5.5	∞	∞
	½	2.5	3.9	6.2	∞
	1	1.4	3.6	8.5	∞
	2	1.6	3.2	2.1	8.2
	3½	1.4	3.3	1.8	4.8
	5	1.4	3.4	1.2	5.6

This table demonstrates that, under the influence of a treatment with Amphenone "B", relatively higher amounts of MIT are found as compared to control animals. (This may also signify that lower amounts of DIT are present in the treated glands.) In this respect some differences between Exp. VII and Exp. VIII are remarkable. In Exp. VII the values for MIT/DIT at ¼ and 1 h. after the injection with NaI^{131} are remarkably higher than in Exp. VIII. First it is mentioned that in Exp. VII the Amphenone was injected 1 h. before the injection of NaI^{131}, whereas in Exp. VIII the time between the two injections only was 10 min. It seems possible to think therefore that in Exp. VIII the Amphenone "B" first not was acting maximally. This perhaps, though to a lesser extent, has caused an initial decrease of MIT/DIT as normally occurs in the untreated animals.

Another fact by which differences may have occurred is the less avidity for radioiodine of the thyroid glands in Exp. VIII, as shown by the contents of total radioiodine (table XX). We will come back to this point in the general discussion of the action of the antithyroid drugs, used in these studies (Chapter VIII). Here also we may claim that the initial high values of MIT/DIT in the treated animals, as compared to the control animals, may be caused by the fact that less iodine can be used for the iodination of amino acids. Due to the less iodine which is available for the iodination of amino acids, it takes a longer time before

a notable amount of DIT is formed. The slight increase in the value of MIT/DIT in Exp. VIII will be discussed in Chapter VIII.

The rate of the inhibition once more can be seen by comparing the protein bound radioiodine in treated and untreated animals (table XXIII).

TABLE XXIII

The percentage of blocking by Amphenone "B" in Exp. VII and Exp. VIII (Proteinbound I^{131} in Amphenone "B"-treated animals as a percentage of that of control animals)

Time after inj. of NaI^{131} in hours	Relative blocking by Amphenone "B" in Exp. VII	Exp. VIII
¼	14.8	32.4
½		13.3
1	18.5	34.0[1]
2		24.2
3½		21.6
5		27.9
7	48.4	

[1] As is seen in table XX the control animals of this time had concentrated a relatively small amount of radioiodine. Therefore it seems that the inhibition at this time is very low. Probably the amount quoted will have to be diminished to about the half-value.

From table XXIII it is apparent that about half an hour after the administration Amphenone "B" in Exp. VIII did not act maximally. Probably the inhibition would have been stronger if the Amphenone "B" had been administered earlier. This is in agreement with the fact that in Exp. VII ¼ h. after the injection with radioiodine the inhibition is about as strong as in Exp. VIII after ½ h. This suggests that after an injection with Amphenone "B" (intramuscularly) the inhibiting effect in the thyroid gland will be maximal about 1 h. after the administration of the drug. The inhibition slightly decreases after that time. This may be caused by the non-retention in the thyroid gland, or destruction of the drug by the thyroid cells.

It seems, by comparison of table XX and table XVI, that Amphenone "B" in the dose used (110 mgs) is not as strong an inhibitor as thiourea (37.5 mgs). This is interesting as LEVER and VAN OVERBEEKE (1956) concluded from histological data that 37.5 mgs thiourea would be equivalent to 110 mg Amphenone "B", if both were administered orally. This controversy may be caused by differences in destruction of Amphenone "B" and thiourea.

VIII. COMPARISON OF THE ACTION OF THE ANTITHYROID DRUGS USED

The antithyroid drugs, used in these investigations, do not act similarly on the thyroid processes. Whereas KSCN (Chapter V) inhibits the

iodide concentrating mechanism, thiourea (Chapter VI) and Amphenone "B" prevent the conversion of iodide to iodine (an oxidation-reaction), and thus the incorporation of the iodine in the thyroglobulin. Possibly, however, more actions in the thyroid processes can be found. Therefore we will study more extensively the effects on the thyroid processes of the antithyroid drugs studied.

This study is facilitated by the fact that the separate experiments were carried out in the same way. They can be divided into two groups. In Exps. III, V and VII the antithyroid drugs were administered 1 h. before the injection with radioiodide. These experiments can be compared with one series of control animals. On the contrary in Exps. IV, VI and VIII the antithyroid drugs were administered 10 min. before the injection with radioiodide. Here in each experiment a series of controls were added.

An impression about the synthesis of the hormones is obtained by comparison of the succeeding values of MIT/DIT. In fig. 10 the data of the Exps. III, V and VII are reproduced. In this figure the value of MIT/DIT of the control animals was subtracted from the values of the MIT/DIT in the treated animals. Evidently MIT/DIT of the KSCN-treated animals after some time comes down to the value of the untreated animals. This happens though KSCN, especially in the first period after the injection with radioiodide, strongly inhibits the iodide accumulation by the gland (table XII). As mentioned before, the initial high values of MIT/DIT most probably are caused by the availability of lower amounts of iodine to iodinate the amino acids in the thyroglobulin. The decrease of the amount of iodide ($I^{127} + I^{131}$) which is accumulated, causes a slowing down of the processes involved in the production of thyroid hormones. This slowing down is shown in the values of MIT/DIT. Apparently the resemblance of MIT/DIT of the KSCN-treated animals with those of the untreated animals towards the end of the experiment, is caused by the fact that the effect of KSCN has ceased at his time (Plate VII, fig. 2). As compared to the untreated animals, MIT/DIT for the KSCN-treated animals decreases faster, in consequence of the presence of lower amounts of KSCN, and thus a decrease in the inhibiting effect of the drug. In conclusion it may be said that KSCN does not directly affect the synthesis of the thyroid hormones. It acts indirectly by lessening the amount of iodide which comes available for these processes.

It may be assumed that an indirect effect, as mentioned above for KSCN, also may be demonstrated after treatment with other antithyroid drugs. Then this will be expected to a lesser extent for Amphenone "B", as this drug, as compared to KSCN, is inferior in inhibiting the accumulation of radioiodine (table XX).

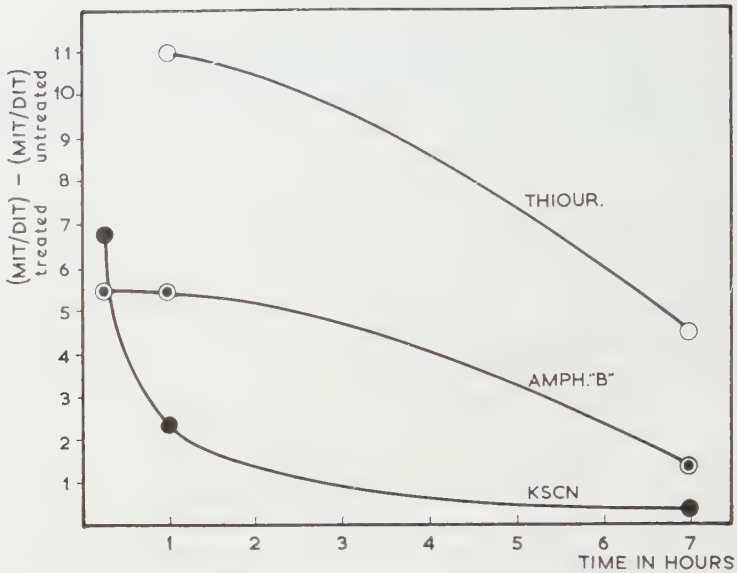

Fig. 10. MIT/DIT values of cockerels, treated with different antithyroid drugs, (MIT/DIT of normal cockerels subtracted) in Exp. III, V and VII

Therefore – if only an indirect effect happened to exist – a less effect on MIT/DIT (on the synthesis of thyroid hormones) as compared to KSCN may be expected. It is shown however (fig. 10) that, especially at 1 and 7 h. after the injection with radioiodide, MIT/DIT decidedly is higher than in the KSCN-treated animals. As mentioned before MIT/DIT may be increased by a decrease of the amount of iodine which is accumulated and is capable of iodinating the amino acids inside the thyroglobulin. This evidently is not the case (though at ¼ h. after the injection of radioiodide this effect may be supposed to exist).

But there remain some other factors which may cause MIT/DIT to be high. First MIT/DIT may be high by an increased proteolysis of the thyroglobulin, as we mentioned in Chapter IV. This, however, also does not fit the case. The proteolytic break-down of the thyroglobulin would happen under the influence of the thyrotropic hormone of the pituitary gland, in consequence of lack of thyroid hormone in the peripheral blood. However this would also happen after treatment with KSCN, being an inhibitor of higher potency. Therefore, most probably, the high value of MIT/DIT may be caused by a direct action of Amphenone "B". Apparently Amphenone "B", beside inhibiting the conversion of iodide to iodine, inhibits also the formation of DIT from MIT. Evidently this inhibition lasts for a longer time than the blocking of the conversion of iodide to iodine. Here also after some time a de-

crease of MIT/DIT sets in, pointing to a decrease of the effect of Amphenone "B".

With respect to thiourea possibly the same holds as for Amphenone "B". Initially no value for MIT/DIT could be determined, as the inhibition of the conversion iodide to iodine was total (table XVIII). Nevertheless after 1 and 7 h. the value of MIT/DIT is very high, as compared to the KSCN-treated animals (fig. 10). It is impossible to explain these high values by an indirect effect upon the synthesis of thyroid hormones, as mentioned for KSCN. Perhaps thiourea also effects directly the formation of DIT (see following pages).

On the ground of the results of these experiments it may be concluded that there exists a strong difference between KSCN on the one hand and Amphenone "B", and probably also thiourea to the other. Whereas KSCN – by way of the effect on the iodide trap – only has an indirect effect on the processes involved in the synthesis of thyroid hormones, by decreasing the amount of iodine which is available to iodinate the amino acids of the thyroglobulin, Amphenone "B", and probably also thiourea, clearly affect these processes – beside the abovementioned indirect effect – directly, by inhibiting to a greater or lesser extent the formation of DIT by iodination of MIT.

We will now have to study, the experiments IV, VI and VIII (see figs. 11 and 12). With respect to the KSCN-treated animals the abovementioned remarks may be quoted. Just the same happens (fig. 11). It is remarkable that the difference in the time of administration of the drug, before the injection with radioiodide, is of relatively small importance. This stresses however the indirect action of KSCN. Though ¼ h. after the injection with NaI^{131} the inhibition of the thyroid gland is not maximal (table XV), clearly there is not, at the time KSCN acts maximally, any shift in the effect upon MIT/DIT. The differences in MIT/DIT in the Exps. III and IV are related to the fact that in Exp. IV the

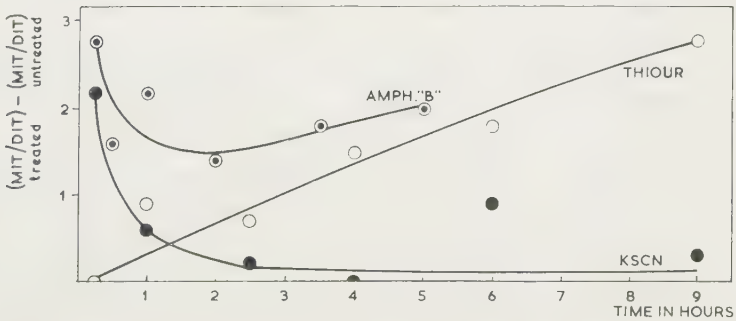

Fig. 11. MIT/DIT values of cockerels, treated with different antithyroid drugs (MIT/DIT of normal cockerels subtracted) in Exp. IV, VI and VIII

physiological activity of the thyroid glands is lower, as demonstrated by a lower content of total radioactivity in the untreated animals of this experiment, as compared with those of Exp. III (table XII). With regard to the Amphenone "B" (Exp. VIII), an initial decrease of MIT/DIT can be observed (fig. 11). At this time however also the inhibition caused by Amphenone "B" is not maximal (table XXIII).

The decrease in the value of MIT DIT can be explained by the normal decrease of this value (Chapter IV), fortified by an indirect effect as mentioned for KSCN. Afterwards however an increase in this value can be noted. This increase can be explained in the same way as in Exp. VII. Here also a direct inhibition of the formation of DIT happens. The results of Exps. VII and VIII are supplementary. In Exp. VII no increase was observed, the physiological activity of the thyroid gland being higher, and the Amphenone "B" being administered at an earlier time, as compared to Exp. VIII. In Exp. VIII however even the beginning of the direct effect of Amphenone "B" can be observed. The differences in the response of the thyroid glands in the two experiments will be caused by a difference in the physiological activity of the glands, by the difference in time of administration of Amphenone "B", and by a relative difference in the dose of Amphenone "B", as the weights of the cockerels in Exp. VII were higher as compared to those of Exp. VIII. By these facts the longer duration of the inhibiting effect in Exp. VIII, as compared to Exp. VII can be explained.

So the results of both experiments are wholly in agreement.

As for thiourea, the data of Exp. VI also are in line with the conclusions obtained from Exp. V. Here also a clear difference with the KSCN-treated animals can be observed (fig. 11). The values of MIT/DIT for the first period after the injection are rather unreliable, as the amounts of radioiodine were very low. In consequence it is impossible to observe any indirect effect as mentioned for KSCN, in the thiourea-treated animals. MIT/DIT increases with time, whereas in the KSCN-treated animals it decreases.

The conclusions, obtained from Exp. V are affirmed: thiourea – beside inhibiting the conversion of iodide to iodine – inhibits also the formation of DIT by iodination of MIT.

Exps. IV, VI and VIII also prove a difference in the effects of KSCN on the one side and of Amphenone "B" and thiourea on the other side upon the synthesis of hormones in the thyroid gland (see fig. 12).

The consequences of the conclusions, drawn from the experiments with antithyroid drugs, for the normal processes involved in the production of hormones in the thyroid glands of birds, now have to be studied.

First evidently the iodide concentrating mechanism has to be seen as

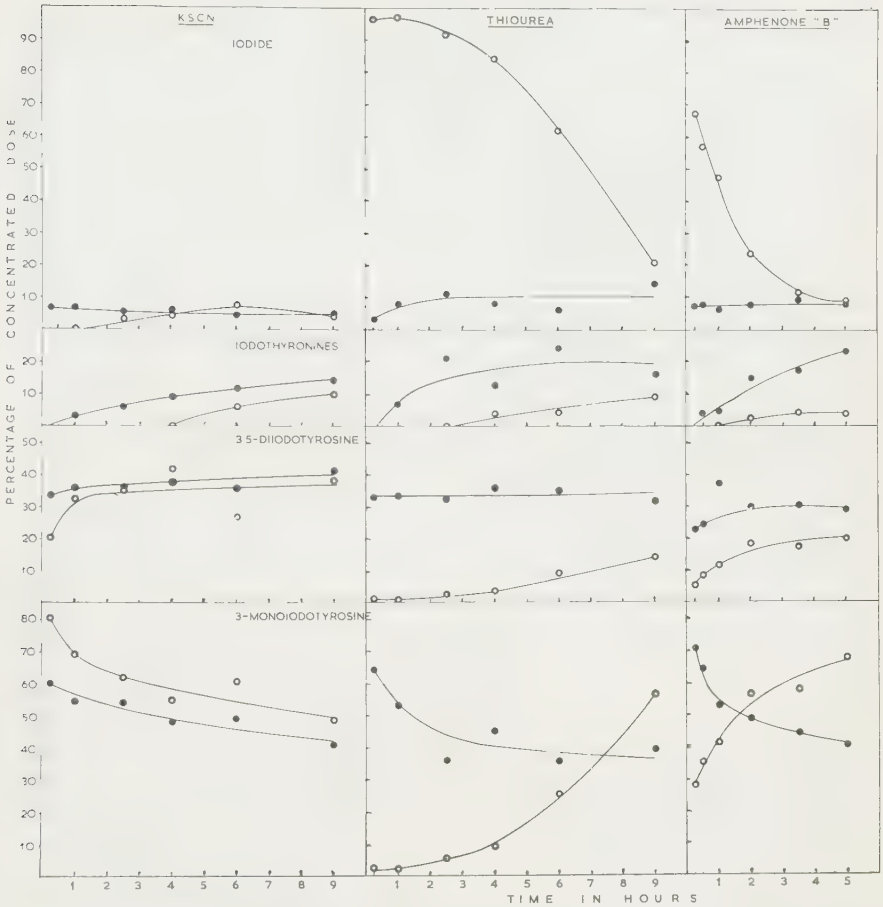

Fig. 12. Radioiodine in the different thyroid fractions of cockerels treated with antithyroid drugs, in percentage of concentrated dose (Exp. IV, VI and VIII)

an autonomous process, as it decidedly has no connections with the iodination and condensation processes which follow it. This is in agreement with the results obtained in mammals (see for instance VANDERLAAN and BISSELL 1946). Secondly the formation of DIT (by iodination of MIT) appeared to be influenced by other factors as compared to the formation of MIT (by iodination of tyrosin), as the formation of DIT can be inhibited by some antithyroid drugs, whereas the synthesis of MIT is not blocked at all. This cannot be caused by a lack of iodine to iodinate MIT, as in KSCN-treated animals, the amount of iodine capable of iodinating tyrosine and MIT also being diminished, no inhibition of the formation of DIT could be observed. This is in keeping

with some data of TAUROG, CHAIKOFF and POTTER (1955). These authors, studying in vitro the iodination of tyrosine by mitochondrial suspensions, also claim the presence of a limiting factor in the formation of DIT, as compared to that of MIT, this limiting factor not being the amount of iodide.

In our experiments, cited above, the value of MIT/DIT was supposed to be a measure for the physiological processes involved in the synthesis of the thyroid hormones. This only is justified at the time that the administered I^{131} is equally distributed among the I^{127}. It is important to study also the partition of the radioiodine among the iodotyrosines as compared to the iodothyronines (Chapter IV). Apparently, however, MIT/DIT also gives a good indication of the mode of action of antithyroid drugs in experiments of relatively short duration.

Clearly with the techniques used, a number of the factors involved in the synthesis of thyroid hormones, could not be detected. So the factors governing the formation of DIT, as compared to MIT, some differences in the two being observed, could not be elucidated. It seems right however, to suppose that any further experiments with the aid of antithyroid drugs, may deepen our insight into the processes involved in the synthesis of hormones in the thyroid gland.

IX. SUMMARY

During the last 15 years, due to the employment of new techniques, our knowledge about the processes involved in the synthesis of hormones in the thyroid gland greatly advanced. Remarkably the investigations were carried out mostly with the aid of mammals as experimental animals. These investigations, as far as the iodinated compounds of the thyroid gland are studied, mostly are of qualitative nature. The experiments carried out in the investigations quoted here, were aimed at elucidating the pattern of the synthesis of thyroid hormones in *birds;* moreover especial attention was devoted to the *quantitative* aspect of these processes. Possibly, in this way differences existing in comparison to other classes of vertebrates (mammals) would be detected. The experiments were carried out with the aid of radioiodine. The different iodinated compounds were analysed by separating them with the help of paper chromatography.

First the maximal content of administered radioiodine in the thyroid gland was found 2–10 h. after the injection. The accumulation of the radioiodine happened to vary rather strongly, not only for each experiment, but also for different groups of one experiment, though in each group 5 animals were pooled. This variability probably is related

to the state of physiological activity of the thyroid gland, which varies not only individually, but also in relation to the season and to the nourishment of the animals. In consequence of some experiments of longer duration, it was claimed that the biological half-time value of the radioiodine within the thyroid gland was about 1 ½–2 days. This is in agreement with the values found for some mammals.

By paper chromatographical analysis of the hydrolysed thyroid material the following iodinated amino acids were demonstrated:

3-monoiodotyrosine (MIT), 3 : 5-diiodotyrosine (DIT), 3 : 5-diiodothyronine (3 : 5-DITh), 3 : 5 : 3′-triiodothyronine (3 : 5 : 3′-TITh) and thyroxine (Tx). Moreover some radioiodine was detected in iodide and to a lesser extent in partly and unhydrolysed thyroglobulin.

In the synthesis of thyroid hormones first of all high amounts of radioiodine were demonstrated in MIT. So it was concluded that this compound is the first step in the processes of synthesis. But also soon rather high amounts are detected in DIT. By comparison of the amounts of radioidine in MIT and DIT (MIT/DIT), with time, the value of this quotient initially appears to be high; rather soon however (during the first experimental hours) MIT/DIT decreases. This is due to the fact that in the beginning DIT only is formed by iodination (with I^{131}) of radioinactive MIT, but afterwards it also is formed by iodination (especially with I^{127}) of radioactive MIT.

About 16–24 h. after the injection the value is 1 (the amounts of radioiodine in MIT and DIT being identical). After that time the value falls below 1 (a higher amount of radioiodine is found in DIT, as compared to MIT).

About 2 h. after the injection radioiodine was demonstrated in 3 : 5 : 3′-TITh and Tx. The amount in 3 : 5 : 3′-TITh (the percentage of radioiodine in this fraction goes up to the utmost to 5% of the total content) is maximal at the time that also in MIT the amount of radioiodine is maximal.

This supports the view that 3 : 5 : 3′-TITh will be formed by condensation of MIT and DIT. Tx only is produced in greater amounts at the moment that a maximum of radioiodine in DIT is demonstrated. This is strongly in favour of the conception that Tx is synthesized by condensation of two molecules of DIT.

The amounts of radioiodine in 3 : 5-DITh continuously are very low. The possibility exists that this is some other iodinated compound, which only in its properties in the chromatographical analysis resembles 3 : 5-DITh.

The amounts of radioiodine in iodide form were very variable. The amounts in this fraction, determined with the aid of the trichloroacetic technique were decidedly lower as compared to the paper chromato-

graphical determinations. This may be caused by adsorptive bindings to proteins of part of the iodide, which are released by hydrolysis of the thyroid material. On the other hand the difference may be caused by a partial deiodination (though to a small extent) of iodinated amino acids. The variability however could not be explained.

From this part of the investigations it is concluded that the synthesis of hormones in the thyroid gland of birds as compared to mammals follows the same pattern.

Differences appear only to a small extent.

The partition of the radioiododine among the different fractions in relation to the amount of the different iodinated ($I^{127} + I^{131}$) amino acids is extensively treated.

A relation only will be detected when the I^{131} in the different fractions equally is spread among the I^{127}.

Then also a constancy in percentages of I^{131} in the different fractions may be expected. This happens to occur at about 20–30 h. after the injection. From this point of time an impression can be obtained about the relative amounts of the different amino acids. It has to be realised however that in Tx 4 atoms of iodine are present, as compared to 1 atom in MIT. So the chance to detect a I^{131}-atom in Tx is 4 × as high as in MIT. Therefore, to obtain the relative quantities of the different amino acids, it is necessary to divide the percentages of radioiodine by the number of I-atoms in the fractions. As the mean values of radioiodine for MIT: DIT: Tx (which also includes the other iodothyronines) are 30 : 40 : 20, the relative amounts of the amino acids are 6 : 4 : 1. By comparing this values with data for mammals (rats) it is claimed that in birds more DIT and less MIT is found.

The abovementioned values are the mean for two experiments. The differences between these two experiments are discussed. It is concluded that, according as more Tx is released into the body (this is caused by a higher state of physiological activity of the thyroid gland), the quotient between iodinated tyrosines and iodinated thyronines is changed on the account of the last.

Furthermore it could be demonstrated that in this case the amount of radioiodine in MIT is higher as compared to DIT. It has to be mentioned that this holds only for normal cockerels, in a period at least 20 hours after the injection with radioiodide.

With the aid of some antithyroid drugs (KSCN, thiourea and Amphenone "B") the processes in the thyroid gland further were analysed. KSCN appeared in birds to inhibit the iodide concentrating mechanism, as was demonstrated in mammals. In MIT/DIT, which was taken as an indicator for the nature and the rate of the hormone synthesis, no qualitative changes were observed. So KSCN has no direct

action on the hormone synthesis. As, however, the amount of iodine, capable for the iodination processes, is diminished by KSCN, surely there exists an indirect action. This causes an increase in the value of MIT/DIT for the treated animals as compared to untreated animals during the first experimental hours. The processes involved in the synthesis of hormones are retarded. Rather soon the value of MIT/DIT falls to the value of the control animals, due to a decrease of the effect of KSCN.

Thiourea in birds, as in mammals, inhibits the conversion of iodide to iodine. Moreover it could be demonstrated, by analysis of MIT/DIT, that thiourea probably inhibits also the synthesis of DIT. Apparently in the synthesis of this compound other factors may be involved than in the synthesis of MIT. With regard to the normally occurring processes it was concluded that the iodide-concentrating mechanism is autonomous with respect to the following iodination and condensation processes, and also that mono-iodination and di-iodination are governed by different factors.

As to Amphenone "B", this compound could be proved to inhibit, exactly as thiourea, the conversion of iodide to iodine. Up to the present the mode of action of this compound was not exactly known. Furthermore also a direct action on the synthesis of DIT, could be demonstrated.

Most likely, by investigations such as those mentioned above, some more points of view can be demonstrated, which will deepen our knowledge about the processes involved in the synthesis of hormones in the thyroid glands.

X. REFERENCES

ADAMS, A. E. and M. F. BENNETT, 1953: Effects of Amphenone "B" on chick embryo's. J. Exp. Zool., **123**, 315–333.

ALBERT, A., 1952: Thyroid gland. Ann. Rev. Physiol., **14**, 481–498.

ALLEN, M. J. and A. H. CORWIN, 1950: Chem. studies on pinacols, obtained from p.-aminoacetophenone and p.-aminopropiophenone. J. Am. Chem. Soc., **72**, 117–121.

ANTHEUNISSE, L. J. and J. LEVER, 1956: I^{131}-accumulation in some invertebrates. Proc. Kon. Ned. Ak. Wetensch. series C, **59**, 562–565.

ASPEREN, K. VAN, 1951: De stofwisseling van het 4-methyl-2 thiouracil na toediening bij jonge hanen. Tijdschr. Diergeneesk., **76**, 133–155.

ASTWOOD, E. B., 1949: Mechanisms of action of various antithyroid compounds. Ann. New York Acad. Sc., **50**, 419–442.

ASTWOOD, E. B., and A. BISSELL, 1944: Effect of thiouracil in the iodine content of the thyroid gland. Endocr., **34**, 282–296.

BARKER, M. H., H. A. LINDBERG and M. H. WALD, 1941: Further experiences with thiocyanates. J. Am. Med. Ass., **117**, 1591–1594.

BARKER, S. B., 1955: The circulating thyroid hormone. The Thyroid, Symp. in Biology, **7**, 74–88.

BARKER, S. B., 1955: Thyroid. Ann. Rev. Physiol., **17**, 417–442.

BERG, O. and A. GORBMAN, 1953: Utilization of iodine by the thyroid of the Platyfish, *Xiphophorus (Platypoecilus) maculatus*. Proc. Soc. Exp. Biol. Med., **83**, 751–756.

BERG, O. and A. GORBMAN, 1954: Normal and altered thyroidal function in domesticated goldfish, *Carassius auratus*. Proc. Soc. Exp. Biol. Med., **86**, 156–159.

BEZEM, J. J., F. BRUNNEKREEFT, M. J. E. ERNSTING, J. LEVER and W. TH. NAUTA, 1949: The distribution of orally administered radio-active methylthiouracil in cockerels. Acta Endocrin., **3**, 151–155.

BLANQUET, P., R. STOLL, R. MARAUD, et L. CAPOT, 1953: Sur l'élaboration de thyroxine par la thyroide de l'embryon de poulet. Bull. Soc. Chim. Biol., **35**, 627–632.

BRAASCH, J. W., A. ALBERT, F. R. KEATING and B. M. BLACK, 1955: A note on the iodinated constituents of normal thyroids and of exophthalmic goiters. J. Clin. Endocrin. Metab., **15**, 732–738.

BRADLEY, W. O., 1951: The effects of certain antithyroid drugs on the uptake of radioactive iodine by the frog thyroid. Biol. Bull., **101**, 62–70.

BRENNER, O., A. B. BLACK and R. GADDIE, 1954: Estimation of the rate of thyroid hormonesecretion in man. Clin. Sc., **13**, 441–451.

CARPENTER, E., J. BEATTIE and R. D. CHAMBERS, 1954: The uptake of I^{131} by embryonic chick thyroid glands in vivo and in vitro. J. Exp. Zool., **127**, 249–269.

DONIACH, I., A. HOWARD and S. R. PELC, 1953: Autoradiography. Progr. in Bioph. and Bioph. Chem., **3**, 1–26.

DONIACH, I. and J. H. LOGOTHETOPOULOS, 1955: Radioautography of inorganic iodide in the thyroid. J. Endocrin., **13**, 65–69.

DRECHSEL, E., 1896: Beiträge zur Chemie einiger Seetiere. II Über das Achsenskelett der *Gorgonia cavolini*. Zeitschr. f. Biol., **33**, 85–107.

ELMER, A. W., 1938: Iodine metabolism and thyroid function. Oxford Med. Publ.

FAWCETT, D. M. and S. KIRKWOOD, 1953: The mechanism of the antithyroid action of iodide ion and of the "aromatic" thyroid inhibitors. J. Biol. Chem., **204**, 787–796.

FINK, K. and R. M. FINK, 1948: The formation of monoiodotyrosine from radioiodine in the thyroid of rat and man. Science, **108**, 358–359.

FLEISCHMANN, W., 1951: Comparative physiology of the thyroid and parathyroid glands. Springfield U.S.A.

FONTAINE, M., J. LELOUP, et M. OLIVEREAU, 1953: Etude histologique et biochimique de la glande thyroïde de l'anguille hypophysectomisée. C. r. Soc. Biol., **147**, 255–257.

FRANKLIN, A.L., I. L. CHAIKOFF and S. R. LERNER, 1944: The influence of goitrogenic substances on the conservation in vitro of inorganic iodide to thyroxine and diiodotyrosine by thyroid tissue with radioactive iodine as indicator. J. Biol. Chem., **153**, 151–162.

FRANKLIN, A. L., S. R. LERNER and I. L. CHAIKOFF, 1944: The effect of thiouracil on the formation of thyroxine and diiodotyrosine by the thyroid gland of the rat with radioactive iodine as indicator. Endocr., **34**, 265–268.

FRETTER, V., 1952: Experiments with P^{32} and I^{131} on species of *Helix*, *Arion* and *Agriolimax*. Quart. J. of Micr. Sc., **93**, 133–136.

GORBMAN, A., 1941: Identity of an iodine storing tissue in an ascidian. Science, **94**, 192–193.

GORBMAN, A., and O. BERG, 1955: Thyroidal function in the fishes *Fundulus heteroclitus*, *F.majalis* and *F.diaphanus*. Endocr., **56**, 86–92.

GORBMAN, A., M. CLEMENTS and R. O'BRIEN, 1954: Utilization of radioiodine by invertebrates, with special study of several Annelida and Mollusca. J. exp. Zool., **127**, 75–89.

GORBMAN, A., S. LISSITZKY, R. MICHEL and J. ROCHE, 1952: Thyroidal metabolism of iodine in the shark *Scyliorhinus (Scyllium) canicula*. Endocr., **51**, 311–321.

GROSS, J. and C. P. LEBLOND, 1951: The presence of free iodinated compounds in the thyroid and their passage into the circulation. Endocr., **48**, 714–725.

GROSS, J., C. P. LEBLOND, A. E. FRANKLIN and J. H. QUASTEL, 1950: Presence of iodinated amino acids in unhydrolyzed thyroid and plasma. Science, **111**, 605–608.

GROSS, J. and R. PITT-RIVERS, 1952 A: Experimental study of thyroid metabolism with radioactive iodine. Brit. Med. Bull., **8**, 136–141.

GROSS, J. and R. PITT-RIVERS, 1952 B: The identification of 3 : 5 : 3′-l.-triiodothyronine in human plasma. The Lancet, **262**, 439–441.

GROSS, J. and R. PITT-RIVERS, 1953 A: 3 : 5 : 3′ triiodothyronine. 1. Isolation from thyroid gland and synthesis. Biochem. J., **53**, 645–652.

GROSS, J. and R. PITT-RIVERS, 1953 B: 3 : 5 : 3′ triiodothyronine; 2. Physiological activity. Biochem. J., **53**, 652–657.

GROSS, J. and R. PITT-RIVERS, 1954: Triiodothyronine in relation to thyroid physiology. Rec. Progr. Horm. Res., **10**, 109–115.

HALMI, N. S. and R. G. STUELKE, 1956: Radioiodine not dischargeable with perchlorate in the thyroids of propylthiouracil treated rats. Endocr., **59**, 134–137.

HALMI, N. S., R. G. STUELKE and M. D. SCHNELL, 1956: Radiodiodine in the thyroid and in other organs of rats treated with large doses of perchlorate. Endocr., **58**, 634–650.

HARINGTON, C. R. and G. BARGER, 1927: III. Constitution and synthesis of thyroxine. Biochem. J., **21**, 169–181.

HEMING, A. E., D. E. HOLTKAMP, J. F. KERWIN, L. F. MANSOR and J. G. DACANAY, 1952: Effect of Amphenone "B" on adrenal and thyroid function of adult ovarioectomized rats. Proc. Soc. Exp. Biol. Med., **80**, 154–157.

HERTZ, R., M. J. ALLEN and W. W. TULLNER, 1950: Effects of Amphenone "B" on thyroid, adrenals and genital tract of the female rat. Proc. Soc. Exp. Biol. Med., **75**, 627–630.

HERTZ, R., M. J. ALLEN, W. W. TULLNER and B. B. WESTFALL, 1952: Surgical anesthesia in rabbit and dog with intravenous Amphenone "B". Proc. Soc. Exp. Biol. Med., **79**, 42–43.

HERTZ, R., J. A. PITTMAN and M. M. GRAFF, 1956: Amphenone toxicity and effects on adrenal and thyroid function in man. J. Clin. Endocr. Metab., **16**, 705–723.

HERTZ, R., W. W. TULLNER and M. J. ALLEN, 1951: Selective inhibition of adrenal and thyroid stimulating effect of Amphenone "B" by cortisone and thyroxine. Proc. Soc. Exp. Biol. Med., **77**, 480–481.

HOFMANN, E., 1950: Thyroxine secretion rate and growth in the White Pekin duck. Poultry Sc., **29**, 109–114.

HOGNESS, J. R., N. D. LEE and R. H. WILLIAMS, 1952: Effects of Amphenone "B" on thyroid and adrenal functions. Endocr., **52**, 378–389.

HOGNESS, J. R., R. H. WILLIAMS and M. LANCE, 1952: Effects of Amphenone "B" on adrenal, thyroid and testes. Proc. Soc. Exp. Biol. Med., **79**, 43–47.

LACHIVER, F. et J. LELOUP, 1955: Sur l'importance du rapport monoiodotyrosine diiodotyrosine dans la biosynthèse des hormones thyroïdiennes. C. r. Soc. Biol., **241**, 573–575.

LAIDLAW, J. C., 1949: Nature of the circulating thyroid hormone. Nature, **164**, 927–928.

LARSON, R. A., F. R. KEATING JR, W. PEACOCK and R. W. RAWSON, 1945: The effect of thiouracil on the collection of radioactive iodine by the thyroid of the chick. Endocr., **36**, 160–169.

LEBLOND, C. P., 1951: The fate of iodine in the body. J. Am. Pharm. Ass., **40**, 595–606.

LEBLOND, C. P. and J. GROSS, 1948: Thyroglobulin formation in the thyroid follicle visualized by the "coated autograph" technique. Endocr., **43**, 306–324.

LEBLOND, C. P. and P. SUE, 1941: Iodine fixation in the thyroid as influenced by the hypophysis and other factors. Am. J. Physiol., **134**, 549–561.

LEIN, A., 1943: Studies on the fixation of radioactive iodine by the rabbit thyroid. Endocr., **32**, 429–432.

LELOUP, J., 1956: Contribution à l'étude du functionnement thyroïdien d'un Teleostéen amphibiotique: *Periophthalmus koelreuteri*. C. r. Acad. Sc., **243**, 1765–1767.

LELOUP, J. et O. BERG, 1954: Sur la présence d'acides aminés iodés (monoiodotyrosine, diiodotyrosine, et thyroxine) dans l'endostyl de l'ammocoete. C. r. Acad. Sc., **238**, 1069–1071.

LELOUP, J. et F. LACHIVER, 1955: Influence de la teneur en iode du régime sur la biosynthèse des hormones thyroïdiennes. C. r. Soc. Biol., **241**, 509–511.

LEVER, J., 1948: A mathematical method for the determination of the state of activity of the thyroid gland. Proc. Kon. Ned. Akad. v. Wetensch., **51**, 1302–1309.

LEVER, J., 1950: Onderzoekingen betreffende de schildklierstructuur. Thesis, Utrecht, 1–133.

LEVER, J. and A. P. VAN OVERBEEKE, 1956: On the antithyroid effect of Amphenone "B" in cockerels. Acta Endocrinol., **22**, 279–282.

LOGOTHETOPOULOS, J. H. and N. B. MYANT, 1956: Concentration of thiocyanate labelled with sulphur-35 in the thyroid of the hamster. Nature, **178**, 646.

MAC QUILLAN, M. T., P. G. STANLEY and V. M. TRIKOJUS, 1954: A study of the action of purified thyroid protease on ^{131}-labelled thyroglobulin. Austr. J. Biol. Sc., **7**, 319–333.

MAC QUILLAN, M. T. and V. J. TRIKOJUS, 1953: Purification and properties of thyroid protease. Austr. J. Biol. Sc., **6**, 617–629.

MICHEL, R., 1950: Contribution à la biochimie des iodoprotéines et des acides aminés iodés. Thèse, Lons-Le-Saunier.

MITSKEVICH, M. S. and Y. A. MAMUL, 1953: Determination of the beginning of function of the thyroid gland in embryos of birds and mammals with the aid of radioactive iodine I^{131}. Dokl. Akad. Nauk. S.S.S.R., **88**, 733–736. (Ex. Med. Endocrin., **7**, 1654, 1953).

MIXNER, J. P., E. P. REINEKE and C. W. TURNER, 1944: Effect of thiouracil and thiourea on the thyroid gland of the chick. Endocr., **34**, 168–174.

MORTON, M. E., I. PERLMAN, E. ANDERSON and I. L. CHAIKOFF, 1942: Radioactive iodine as an indicator of the metabolism of iodine. Endocr., **30**, 495–501.

NADLER, N. J., C. P. LEBLOND and R. BOGOROCH, 1954: The rate of iodine metabolism by the thyroid follicle as a function of its size. Endocr., **54**, 154–172.

NANDI, S., R. K. PODDAR and C. K. PYNE, 1955: The total and differential concentration of radio-iodine in thyroid tissue and its variation with time, as studied by auto-radiography. J. Endocrin., **13**, 125–132.

PITT-RIVERS, R., 1948: The oxidation of diiodotyrosine derivatives. Biochem. J., **2**, 223–231.

PITT-RIVERS, R., 1948: The action of antithyroid substances on the formation in vitro of acetylthyroxine from acetyldiiodotyrosine. Bioch. Biophys. Acta, **2**, 311–318.

PITT-RIVERS, R., 1950: Mode of action of antithyroid compounds. Physiol. Rev., **30**, 194–205.

PITT-RIVERS, R. and W. R. TROTTER, 1953: The site of accumulation of iodine in the thyroid of rats treated with thiouracil. Lancet, **265**, 918–919.

RABEN, M. S., 1949: The paradoxical effects of thiocyanate and of thyrotropin on the organic binding of iodine by the thyroid gland in the presence of large amounts of iodide. Endocr., **45**, 296–304.

RAWSON, R. W., J. F. TANNHEIMER and W. PEACOCK, 1944: The uptake of radioactive iodine by the thyroids of rats made goiterous by potassium thiocyanate and by thiouracil. Endocr., **34**, 245–253.

ROBERTIS, E. de, 1941: Proteolytic enzyme activity of colloid extracted from single follicles of the rat thyroid. Anat. Rec., **80**, 219–231.

ROBERTIS, E. de, 1954: Physiological hydrolysis of thyroglobulin in the thyroid gland. Acta Phys. Lat. Am., **4**, 77–92.

ROCHE, J., 1952: Biochimie comparée des scléroprotéines iodées des Anthozoaires et des Spongiaires. Experientia, **8**, 45–54.

ROCHE, J., S. LISSITZKY et R. MICHEL, 1951: Caractérisation de la monoiodohistidine et de la diiodothyronine dans la thyroglobuline marquée. C. r. Acad. Sc., **232**, 2047–2049.

ROCHE, J., S. LISSITZKY et R. MICHEL, 1952: Sur la triiodothyronine, produit intermédiaire de la diiodothyronine en thyroxine. C. r. Acad. Sc., **234**, 997–998.

ROCHE, J., S. LISSITZKY et R. MICHEL, 1952: Caractérisation des iodohistidines dans les protéines iodées (thyroglobuline et iodoglobuline). Bioch. Biophys. Acta, **8**, 339–345.

ROCHE, J., O. MICHEL, R. MICHEL, A. GORBMAN et S. LISSITZKY, 1953: Sur la déshalogénation enzymatique des iodotyrosines par le corps thyroïde et sur son rôle physiologique. Biochim. Biophys. Acta, **12**, 570–576.

ROCHE, J., O. MICHEL, R. MICHEL et S. LISSITZKY, 1953: Sur la déshalogénase thyroïdienne. C. r. Soc. Biol., **147**, 232–234.

ROCHE, J. et R. MICHEL, 1951: Thyroglobuline, protéines artificiellement iodées et hormone thyroïdienne. Annales d'Endocrin., **12**, 317–332.

ROCHE, J. and MICHEL, 1954: Thyroid hormones and iodine metabolism. Ann. Rev. Bioch., **23**, 481–500.

ROCHE, J. and R. MICHEL, 1955: Nature, biosynthesis and metabolism of thyroid hormones. Physiol. Rev., **35**, 583–610.

ROCHE, J. and R. MICHEL, 1956: Nature and metabolism of thyroid hormones. Rec. Progr. in Horm. Research, **12**, 1–26.

ROCHE, J., R. MICHEL et S. LISSITZKY, 1951: Déshalogénation enzymatique des acides aminés iodés dans le corps thyroide et thyroxinémie. C. r. Soc. Biol., **145**, 288–290.

ROCHE, J., R. MICHEL, J. NUNEZ et W. WOLF, 1955: Sur deux constituants nouveaux du corps thyroide: la 3 : 3′-diiodothyronine et la 3 : 3′ : 5′-triiodothyronine. Biochim. Biophys. Acta, **18**, 149–150.

ROCHE, J., R. MICHEL et W. WOLF, 1955: Présence probable de la 3 : 3′ : 5′ triiodothyronine dans a thyroglobuline. C. r. Acad. Scienc., **240**, 251–253.

ROCHE, J., R. MICHEL, W. WOLF et J. NUNEZ, 1956: Sur deux nouveaux constituants hormonaux du corps thyroide, la 3 : 3′-diiodothyronine et la 3 : 3′ : 5′-triiodo thyronine. Biochim. Biophys. Acta, **19**, 308–317.

ROSENBERG, I. N., 1952: The antithyroid activity of some compounds that inhibit peroxidase. Science, **116**, 503–505.

SHELLABARGER, C. J., A. GORBMAN, F. C. SCHATZLEIN and D. MCGILL, 1956: Some quantitative and qualitative aspects of I^{131} metabolism in turtles. Endocr., **59**, 331–339.

STANLEY, P. G., 1953: Artefacts produced from 3-iodo-tyrosine and 3 : 5 di-iodotyrosine by treating with alkali. Nature, **171**, 933–934.

STOLL, R. et P. BLANQUET, 1952: Sur l'activité des thyroides de l'embryon de poulet, étudiée à l'aide du radioiode I^{131}. Ann. d'End., **13**, 569–577.

STOLL, R., P. BLANQUET et R. MARAUD, 1954: Sur l'activité de la thyroide chez l'embryon de certaines galliformes, étudiée à l'aide d'I^{131}. Ann. d'End., **15**, 172–177.

TAUROG, A. and I. L. Chaikoff, 1947: On the nature of plasma iodine. J. Biol. Chem., **171**, 439–440.

TAUROG, A., I. L. CHAIKOFF and D. D. FELLER, 1947: The mechanism of iodine concentration by the thyroid gland: its non-organic iodine binding capacity in the normal and propylthiouracil-treated rat. J. Biol. Chem., **171**, 189–201.

TAUROG, A., I. L. CHAIKOFF and A. L. FRANKLIN, 1945: The structural specificity of sulfanilamide-like compounds as inhibitors of the in vitro conversion of inorganic iodide to thyroxine and diiodotyrosine by thyroid tissue. J. Biol. Chem., **161**, 537–543.

TAUROG, A., G. D. POTTER, and I. L. CHAIKOFF, 1955: Conversion of inorganic I^{131} to organic I^{131} by cell-free preparations of thyroid tissue. J. Biol. Chem., **213**, 119–134.

TAUROG, A., W. TONG and I. L. CHAIKOFF, 1950: The monoiodotyrosine content of the thyroid gland. J. Biol. Chem., **184**, 83–97.

THOMAS, J. M., 1956: The accumulation of radioactive iodine by Amphioxus. J. Mar. biol. Ass. U. K., **35**, 203–210.

TRUNNELL, J. B. and P. WADE, 1955: Factors governing the development of the chickembryo thyroid. J. Clin. End. Metab., **15**, 107–117.

VANDERLAAN, J. E. and W. P. VANDERLAAN, 1947: The iodide concentrating mechanism of the rat thyroid and its inhibition by thiocyanate. Endocr., **40**, 403–416.

VANDERLAAN, W. P. and A. BISSELL, 1946: Effects of propylthiouracil and of potassium thiocyanate on the uptake of iodine by the thyroid gland of the rat. Endocr., **39**, 157–160.

VANDERLAAN, W. P. and V. M. STORRIE, 1956: A survey of the factors controlling thyroid function, with especial reference to newer views on antithyroid substances. Pharmacol. Rev., **7**, 301–334.

VLIJM, L., 1956: The effects of Amphenone "B" on the I^{131}-accumulation in the thyroid gland of cockerels, compared with that of KSCN and thiourea. Acta Endocrinol., **12**, 273–278.

WHEELER, B. M., 1950: Halogen metabolism of *Drosophila gibberosa*. Part I. Iodine metabolism studied by means of I^{131}. J. exp. Zool., **115**, 83–107.

WOLFF, J. and I. L. CHAIKOFF, 1947: The relation of thyroxine to total iodine in the thyroid gland. Endocr., **41**, 295–298.

Wolff, J., I. L. Chaikoff, A. Taurog and L. Rubin, 1946: The disturbance in iodine metabolism produced by thiocyanate: the mechanism of its goitrogenic action with radioactive iodine as indicator. Endocr., **39**, 140–148.

Wollman, S. H. and I. Wodinsky, 1955: Localization of protein-bound I^{131} in the thyroid gland of the mouse. Endocr., **56**, 9–20.

Wollman, S. H. and E. Zwilling, 1953: Radioiodine metabolism in the chick embryo. Endocr., **52**, 526–535.

Wolstenholme, G. E. W. and E. C. P. Millar, 1957: Colloquia on endocrinology, (CIBA foundation), **10**, Regulation and mode of action of thyroid hormones, London.

Wood, J. L. and N. Kingsland, 1950: Labeled sulphur uptake by thyroids of rats with low plasma thiocyanate levels. J. Biol. Chem., **185**, 833–837.

Wijngaarden, J. B., B. M. Wright and P. Ways, 1952: The effect of certain anions upon the accumulation and retention of iodide by the thyroid gland. Endocr., **50**, 537–549.

THE INNERVATION OF THE MUSCLES OF THE TRAPEZIUS-COMPLEX IN GIRAFFE, OKAPI, CAMEL AND LLAMA

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INTRODUCTION

The present paper is concerned with the innervation of the m.trapezius, the m.omocervicalis, the m.cleidocephalicus and the m.sternocephalicus in long-necked Ungulates. These muscles usually are innervated by the n.accessorius spinalis. This nerve arises from the cervical part of the spinal cord. The fibres run in anterior direction along the spinal cord. They enter the skull through the foramen magnum and leave again through the jugular foramen together with the bulbar part of the n.accessorius and the n.vagus. In the vicinity of the ala atlantis the nerve splits into a ventral and a dorsal branch. The r.dorsalis supplies the m.trapezius and the m.cleidocephalicus. The m.sternocephalicus is innervated by the r.ventralis. In addition the muscles of the trapezius-complex are supplied by afferent branches from cervical nerves. In the above-mentioned Ungulates, belonging to quite different systematical groups, the literature indicates that there are variations from the usual way of innervation of the trapezius-complex.

SLIJPER (1947) states that in long-necked Ungulates (camel, llama and giraffe) the whole trapezius-complex is exclusively innervated by cervical nerves. To justify his statement, partially based on data in the literature, this author induced the investigation of the innervation of the trapezius-complex in available specimens of the proper Ungulates at the Zoological Laboratory of the University of Amsterdam.

We shall present our own findings in the different kinds of animals in comparison with the most important data in the literature.

GIRAFFE, GIRAFFA CAMELOPARDALIS (L.)

ZUCKERMAN and KISS (1932) made an attempt to obtain certainty about the spinal accessory nerve of the giraffe. At first they give us the data in the literature at hand at that moment. Quoting TODD and TODD (1912) they write that "in the camels, the llamas and the giraffes the spinal portion of the accessory is entirely absent and the muscles of the trapezius-complex obtain both their afferent and efferent supply from the cervical nerves". On the other hand VERMEULEN (1918) states that in a preparation of the first two cervical segments of the spinal cord the nervi accessorii Willisii could be seen perfectly intact in their usual course between the roots of the first two cervical nerves.

The dissections of two giraffes, carried out by ZUCKERMAN and KISS themselves, indicate that the muscles of the trapezius-complex were supplied, as in other Ungulates, by branches from the spinal accessory and from cervical nerves.

The dissection of a giraffe at our own laboratory gave results which resembled those of ZUCKERMAN and KISS very much. As in their specimens the relations of the nerve near the jugular foramen were in no respect different from those in other Mammals. In its course along the neck the nerve is more superficial than in any other Mammal and can easily be removed while skinning. This superficial course is partially caused by the lacking of the major part of the m.cleidocephalicus. The remaining p.cleidocervicalis was innervated by cervical nerves from C_V, C_{VI}, and C_{VII}. The n.accessorius supplied the m.trapezius.

OKAPI, OKAPIA JOHNSTONI SCLATER

This year we had the rare opportunity of dissecting an okapi which died in the Zoological Garden "Artis". As in the giraffe the n.accessorius was present in the okapi. The relation of the nerve at the base of the skull could not be studied for the reason that the head had already been promised for other investigations. At the ventral border of the ala atlantis the nerve splits into a r.dorsalis and a r.ventralis. The course of the r.dorsalis along the neck is very normal and less superficial than in the giraffe. This branch continues its course to the m.trapezius which is innervated. The r.ventralis supplies the m.sternomandibularis. Contrary to the former muscles the m.cleidocervicalis is supplied by cervical nerves. Many fibres of C_{IV}, C_V, C_{VI} and C_{VII} enter this muscle and apparently do not leave it again.

CAMEL, CAMELUS BACTRIANUS L.

As Kiss and Zuckerman showed already, the n.accessorius was present in the giraffe, so they welcomed the opportunity to dissect a camel (1934). They did not find an external branch of the spinal accessory. The muscles of the trapezius-complex were innervated by cervical nerves. However a spinal part of the n.accessorius was present, extending downwards as far as the second cervical nerve. The fibres were lying, as is usual, between the ventral and dorsal roots of the spinal nerves. Contrary to the relations in most Mammals this small spinal accessory joined the vagus, as the bulbar part always does, and could not be examined any farther.

We dissected three camels ourselves. In all specimens the m.trapezius, the m.omocervicalis and the m.cleidocervicalis were innervated by cervical nerves. The m.trapezius and m.omocervicalis got their supply from fibres from C_{VII}, the m.cleidocervicalis from C_{VI} and C_{VII}. In one camel, a neonatus, a branch of C_{VI} was found, extending to the cranial part of the m.trapezius.

Contrary to the two adult camels, in the neonatus we had the opportunity to dissect the head and anterior part of the neck in order to obtain information about the origin of the spinal accessory. The relations found in that area were quite the same as described by Kiss and Zuckerman.

An interesting problem that has had our attention for a long time concerns the conditions of the n.recurrens in the camel. This nerve does not participate in the innervation of the muscles of the trapezius-complex, but is important in connection with the present investigations because of its inefficient course. Usually it arises as a branch from the n.vagus in the anterior part of the thorax; turning around the a.subclavia at the right side and the ductus arteriosus Botalli at the left, running forward on the ventral surface of the trachea and ascending the neck along the common carotid artery. If the lacking of the n.accessorius in the camel is caused by the length of the neck, one would expect that the n.recurrens is influenced too. However in the young camel we dissected a very normal n.recurrens was present.

LLAMA, LAMA GLAMA (L.)

Howell and Strauss (1934) describe the dissection of a llama. In this Ungulate the muscles of the trapezius-complex were innervated by cervical nerves. Besides the m.sternomandibularis was supplied by a nerve that emerged from the vicinity of the jugular foramen. At

first sight it was considered as an external branch of the n.accessorius but upon examining the upper cervical region of the spinal cord it was discovered that the spinal origin of the n.accessorius was absent. Since it was impossible to dissect the head, the origin of the branch could not be studied. In our animals the muscles mentioned were innervated by cervical nerves. A study of the head and the anterior part of the neck was not attempted.

CONCLUSION

The statement made by SLIJPER — in long-necked Ungulates the whole trapezius-complex is exclusively innervated by cervical nerves — is incorrect. Some twenty years ago anatomists showed that in the giraffe a n.accessorius is present, but the nerve is lacking in camels and llamas. Recent investigations are in accordance with these facts.

There still remain a number of interesting questions: Is the lacking of the nerve in camels caused by the length of the neck? The existence of the n.accessorius in the giraffe and the presence of a normal n. recurrens in the camel do not agree with a great influence of the length of the neck. In this connection the question arises: What relations are found in long-necked birds? No one ever answered this question.

Maybe the aberrant course of the efferent supply of the muscles of the trapezius-complex in the camel is caused by the time of development of the different organs in the embryo. We know that the dorsal elements of the spinal cord are developed later than the ventral ones. Moreover we know that the muscle-mass of the trapezius-complex passes in caudal direction along the neck during ontogenesis. From this point of view it is understandable that the afferent fibres supplying the muscles usually originate from cervical roots, supposing that the nerve fibres take a course as short as possible to the muscles already formed. Further we may expect that the development of the efferent supply takes place at a later — or the development of the muscles at an earlier — stage in the embryo of the camel, compared with the relations in other Mammals.

It would be very desirable to study young embryos of the camel, but so far this was not attempted.

LITERATURE

Howell, A. B. and W. L. Strauss, 1934: Note on the spinal accessory nerve of the long necked Ungulates. Proc. Zool. Soc. London, 29.

Kiss, F. and S. Zuckerman, 1934: The spinal accessory nerve of the camel. Proc. Zool. Soc. London, 71.

Slijper, E. J., 1947: In Ihle *et al.*, Leerboek der vergelijkende ontleedkunde der Vertebraten, Utrecht.

Strauss, W. L. and A. B. Howell, 1936: The spinal accessory nerve and its musculature. Quart. Rev. Biol., **11**, 387.

Zuckerman, S. and F. Kiss, 1932: The spinal accessory nerve of the giraffe. Proc. Zool. Soc. London, 767.

ANNUAL REPORT OF THE ZOOLOGICAL STATION OF THE NETHERLANDS ZOOLOGICAL SOCIETY FOR THE YEAR 1956

If we consider the work that has been done at Den Helder during the last decades, it appears that much time and effort has been devoted to the ecology of molluscs. In the beginning attention was mainly concentrated on the factors governing distribution or abundance after settling. Gradually, the trend of activity was more and more directed to larval ecology and the settling of the spat. This part of the research presents serious difficulties so that the results do not always correspond with the laborious efforts spent upon these items. Still, it seems worth while to proceed along this line and, especially, to find out in how far the larvae are active or passive with regard to their own conveyance. As a matter of fact, students again and again take an interest in this item. In 1956 it was Mr. J. W. F. Nuboer from Utrecht University, who continued previous attempts, especially to find out whether spat which are about to settle might prefer a certain degree of illumination and therefore a certain depth of water. He spent much time in technical preparations, and we hope the work will be continued in 1957.

Mr. Dral resumed the investigations about the pumping and the straining of food in mussels, previously undertaken by him in 1952 under the supervision of Dr. Tammes. This subject has reached a stage in which it becomes hard to make further progress. In the previous annual report it was explained that mussels can probably control the retention of food by regulating the position of the laterofrontal cilia. Now, Mr. Dral wondered in what way the position of the abovementioned cilia could be changed. He was inclined to suppose that some minute muscles, which are known to exist in the filaments of the gills, could by relaxation alter the frontside of the filaments so as to give the laterofrontal cilia a different position, at the same time widening the ostia between the filaments, thus restricting retention. Hoping to demonstrate these changes in the filaments and the width of the ostia, Mr. Dral administered adstringent and relaxing drugs. Subsequent

fixation and histological examination of the tissues did not, however, yield any evidence to support his views. This experience, as well as the difference in the action of the laterofrontal and lateral cilia, made it desirable to resort to the electron-microscope. To this end cooperation was established with the Laboratory for Electron-Microscopy at Leyden, where Mr. BRAAMS and his collaborators not only offered great help, but even took a personal interest in the subject. Mention should further be made of motion pictures made of the movement of the gill cilia. They form part of a film about the mussel and its culture. This film was made by Mr. WINDMEYER (Delft) by order of the Department of Fisheries. In a part of it also "University Film" (Utrecht) cooperated. The "Netherlands Educational Films" (The Hague) has also been helpful to us in making a motion picture of the feeding mechanism of the mussel. Apart from this, Mr. DRAL spent much time in working out data, collected earlier, on the influence of temperature and other factors on the pumping of mussels. We hope that Mr. DRAL's observations will appear in a paper in the Archives, possibly in 1959.

Mr. KRISTENSEN collected a number of observations on the effects of the severe and prolonged cold of the winter 1955/'56, which was, as might be expected, catastrophic to all sessile animals within the tidal area. On account of previous experience Mr. KRISTENSEN expected that during the following summer the depopulated areas would be densely populated by a new generation, but the density of spat of various molluscs, worms and crustaceans was below normal. This was probably due to the extremely low temperatures in the spring and the summer of this year.

Mr. KRISTENSEN futher completed his investigations on the cockle by collecting some additional data. His work on this species comprises a 10 year period, and his thesis on the subject will soon appear in the "Archives". It is mainly about the influence of external factors on population density and growth of the species, but at the same time makes an important contribution to our knowledge of the fauna of the Wadden area.

In the above mentioned period Mr. KRISTENSEN also paid attention to several molluscspecies of the North Sea. Some details of his observations will be given here.

The presence of a number of Atlantic species in the North Sea was notable in the post war period, especially during the warm season of 1947. It was discontinued during the last two years. The annual growth rate of these species, *Pecten maximus*, *Cardium crassum* and *Dosinia exoleta*, appeared to be quite normal, although the specimens lived near the boundary of their area of distribution here. The data of *Dosinia* were published in De Levende Natuur, **59**, p. 82–84, 1956. An examination

of the shells of the razorclam, *Ensis siliqua*, revealed that the conspicuous difference in size of those washed ashore on the Dutch Westcoast and those washed ashore along the North Sea coast of the Wadden Islands, depends on their age. The specimens of the Dutch coast appear to have a life span of a few years only, with a maximum length of 14 cm, whereas ages of over ten years, with a length of 20 cm, were observed in the shells from the North Sea beaches of the Wadden Islands. Since *Ensis siliqua* is known to be susceptible to low temperatures, Mr. KRISTENSEN assumes that in winter the animals are more likely to die along the shallow Dutch West coast than in the deeper waters north of the Wadden Islands. The data in question were published in De Levende Natuur, **60**, p. 93–95, 1957.

The work on the common cuttle, *Sepia officinalis*, initiated by Miss GEHRELS in 1955, was taken up again by Mr. H. L. DE BEAUFORT, of Utrecht University. In the previous report mention has already been made of our intention to trace the influence of several factors that will make *Sepia* develop a certain colour pattern. Miss GEHRELS' experiments, inspired by work of HOLMES (Proc. Zool. Soc. London, **110**, p. 17–35, 1940), had shown that the presence of white in the surroundings of the cuttle will stimulate the animal's inclination to display a characteristic white patch on its back. It was Miss GEHRELS' opinion that yellow would also exert a significant influence, even more than white. This might indicate that the quality of the light plays a part in causing a reaction. The experiments of Mr. DE BEAUFORT have shown, however, that it is rather the quantity of light that is decisive for its effect on *Sepia*. The experiments will perhaps be continued in the near future. If not, they will be published in the Archives.

Mr. R. NIEUWENHUYS, from the Central Institute for Brain Research, Amsterdam, stayed at Den Helder for some time, in order to make preparations of the so called cauda equina of several kinds of fishes. Mr. NIEUWENHUYS is especially interested in the functional side of the matter.

Mr. R. J. VAN BIEZEN, of the University of Amsterdam, made, by mutual arrangement with Prof. J. TEN CATE and Dr. A. PUNT, an effort to estimate the influence of temperature on the osmotic pressure of the blood of *Carcinus maenas*, using the method of freezing point determination. Mr. VAN BIEZEN had to cope with many technical difficulties, however, so that no results were obtained.

Miss A. J. WARBURG, of Groningen University, decided to choose a subject of research that had for many years been recommended at Den Helder, but until then nobody had chosen it. The slug *Aeolidia papillosa* is known greatly to prefer the sea anemones *Metridium senile* and *Actinia equina* for its food, but there are indications that other

species of anemones are occasionally taken as well. Some observations suggested the possibility that the preference for a certain anemone might not be innate, but acquired in the prime of life. In fact, it was supposed that the newly hatched young slug could perhaps be conditioned to *Diadumene cincta*, with the result that a population would come into being living on *Diadumene cincta*, in spite of the fact that the slug species as a whole does not feel attracted to this anemone. Contrary to our expectations Miss WARBURG found that, apparently, all individuals of *Aeolidia papillosa* accept *Diadumene cincta*, but if given the choice between *Metridium* and *Diadumene* the relation in which both are taken is about 7:1. No individuals were found wholly to refuse *Diadumene*. Spawn of slugs that had been shown to prefer *Metridium* to *Diadumene* was now placed in aquaria with *Diadumene*, in the hope that the young animals, after larval life, would feed on *Diadumene*, and later on give young ones that would prefer *Diadumene* to *Metridium*. The crucial point in these experiments was whether the spat could be kept alive through metamorphosis. *Aeolidia papillosa* is believed to possess a short larval life. It was found, however, that the larval life of this species is less short than it is supposed to be and Miss WARBURG did not succeed in rearing the larvae through metamorphosis. We hope that the subject will be taken up again later on.

Mr. DE BLOK, commissioned by the Netherlands Organization for Pure Research, continued his work concerning lunar and tidal influences on the reproductive rhythm of marine animals. Throughout the year 1956 he has been busy in bringing his complex apparatus into working condition. We hope the influence of several factors, e.g. light, pressure, and so on, can now be studied separately with this installation. Since his program of work has already been mentioned in the previous report, it is not going to be discussed this time. Mention should, however, be made of the fact that L. HAUENSCHILD, Tübingen (Naturwissenschaften, **43**, p. 361–363, 1956) has succeeded in demonstrating that the reproductive rhythm in *Platynereis Dumerilii* is regulated by the period of illumination. He has succeeded in imposing a certain rhythm upon the animals by submitting them to different periods of illumination.

Mr. F. CREUTZBERG, also commissioned by the Organization for Pure Research, continued his research on the orientation of migrating eels. In last year's report it was already explained that his work comprises observations at sea as well as experiments in the laboratory. For these experiments a staging had been constructed on the top of the roof of the Zoological Station; it will enable Mr. CREUTZBERG to find out whether elvers use the sun for orientation. Birds are known to find their way with the help of the sun, but in the case of eels this is still

quite uncertain. Apart from this, observations were made on board of two of the Dutch lightvessels. Regular hauls with nets of coarse plankton gauze have shown that the first appearance of elvers and their subsequent increase in numbers occurs at about the same time off the Belgian coast and near Den Helder. No clue was obtained to ascertain whether the majority reach the Netherlands by passing the Straits of Dover or by rounding the Shetlands, and from the time of migration no conclusions could be drawn as to their passivity or activity during their transport. In another respect, however, the observations did yield important evidence. It had previously been taken for granted that the elvers would only enter the North Sea if the temperature exceeded a certain critical value, but during the period of severe cold they were caught in increasing numbers at water temperatures below zero. The catches have also shown that at night the elvers are found in greater numbers near the surface during flood tide than during ebb tide. Mr. CREUTZBERG also made some cruises on the Wadden Sea, by night towing a coarse plankton net just below the surface, with the current or facing it. Here, too, the numbers caught during the flood were much greater than those caught during the ebb. Hauls of equal duration, however, if made during one and the same tide, yielded equal catches, irrespective of the direction in which the hauls were made. The transport therefore appears to be fully passive, but it takes place during flood only, and, at least near the surface, not during ebb. The observations give an interesting outlook and this line of research will be carried on in 1957.

Mr. WESTENBERG continued to develop his theoretical considerations on population systems. His contribution to the summer course was published in Vakblad voor Biologen (**36**, p. 41–53, 1956). The difference between the structure of the parasite–host relation and predator–prey relation, which was touched upon in this paper, was further discussed in a lecture delivered at a meeting of the ecological section of the Netherlands Zoological Society at Utrecht. On this occasion he also demonstrated that it is not self-evident to introduce constant environmental conditions in a population model.

Mr. KRISTENSEN continued his observations on migratory species by buying up specimens from the fishermen. The year 1956 brought a late, but severe winter; spring and summer were cold as well, whereas the remaining months were rather warm.

The low temperatures of the spring of this year delayed by 2 or 3 weeks the arrival of southern migrants that usually reach our neighbourhood in April and May. With some species this even resulted in an area of distribution different from the normal one. *Sepia* normally has its north boundary near Den Helder, but in 1956 the species

Species	*Sex; Size (cm)*	*Locality (ST means buoy on the Silverpit-Texel-route*
MIGRANTS SUPPOSED TO HAVE ENTERED THE NORTH SEA VIA DOVER STRAIT		
Cetorhinus maximus	♂ 377	Wadden Sea
Torpedo marmorata	♀ 50	15 km E of ST 3 buoy
Raia brachyura	♀ 94	Black Bank
Raia montagui	33–64	Texel Hole-ST2- N. of Terschelling
Spondyliosoma cantharus	33	near ST 4 buoy
Trigla cuculus	21–33	off Texel, and Black Bank
Trigla lineata	24; 31	Black Bank; ST 3 buoy
Sepia officinalis	9–23	mostly from coastal waters and Wadden Sea
Octopus vulgaris		Texel Hole area-N of Terschelling, 40–45 m
MIGRANTS SUPPOSED TO HAVE ENTERED THE NORTH SEA VIA THE NORTHERN ENTRANCE		
Scomberesox saurus	37; ♀ 37	stranded near Den Helder
Brama raii	55; 47; ♂ 50; ♀ 51; 50; 45; 45; 53	nos. 2 & 3: 58° 40′ N 3°20′E; no. 8: off Callantsoog, 9 m; the others stranded between Katwijk and Texel
Mola mola	±85; ±75; ±100	stranded between Egmond and Frisian coast
NORTHERN SPECIES, RARE IN THE S. NORTH SEA		
Gadus poutassou	± 22	W. of Texel, 9 m
Onos cimbrius	± 12	off Callantsoog, 9 m
Anarhichas lupus	29– ± 50	W of Den Helder-N of Terschelling, 28–38 m
Eledone cirrhosa		Texel Hole; N of Terschelling
SPECIES, WHOSE DIRECTION OF MIGRATION IS UNCERTAIN		
Petromyzon marinus	± 65	Wadden Sea
Acipenser sturio	± 150; ± 250	near LV Terschellingerbank; Wadden Sea
Raniceps raninus	7; 8; 8; 8; 6	near Den Helder, 10–20 m
Spinachia spinachia		Harbour of Den Helder
Scorpaena dactyloptera	± 15; 17	E of ST 3 buoy; Pit 3 buoy
Labrus berggylta		off Petten, 21 m
Todarodes sagittatus	24	stranded near Den Helder
Illex illecebrosus		Texel Hole, 35 m

					Numbers per month						
J	F	M	A	M	J	J	A	S	O	N	D
—	—	—	—	1	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	1	—	—
—	—	1	—	—	—	—	—	—	—	—	—
5	1	—	—	1	2	—	—	5	6	5	—
—	—	—	—	—	—	—	—	1	—	—	—
—	—	—	—	2	9	2	1	—	—	—	—
—	—	—	—	—	1	—	1	—	—	—	—
—	—	—	—	2	21	19	3	—	7	1	—
3	—	—	—	—	—	—	—	—	—	—	4
1	—	—	—	—	—	—	—	—	—	—	1
2	2	—	—	—	—	—	—	—	—	—	4
—	—	—	—	—	—	—	—	—	—	—	3
—	1	—	—	—	—	—	—	—	—	—	—
—	—	—	1	—	—	—	—	—	—	—	—
—	1	5	3	3	—	—	—	—	—	—	—
—	—	1	—	—	—	—	—	—	—	—	1
—	—	—	—	1	—	—	—	—	—	—	—
—	1	—	—	—	—	—	1	—	—	—	—
1	—	—	3	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	1	—	—
—	—	1	1	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	1	—	—
—	—	—	—	1	—	—	—	—	—	—	—
—	—	—	—	—	1	—	—	—	—	—	—

scarcely reached us, and remained in the estuarine area of the province of Zeeland. Other southern migrants, too, were strikingly scarce near Den Helder in the first part of the summer. This applies to *Loligo vulgaris*, the rays *Raia brachyura* and *R. montagui*, the pilchard *(Clupea pilchardus)*, the dory *(Zeus faber)*, the red mullet *(Mullus surmuletus)* and to the black bream *(Spondyliosoma cantharus)*. Only the stingray *(Trygon pastinaca)*, which is also a migrant from the South, but usually arrives late in the season, was fairly numerous in 1956.

The migrants that as a rule pass Den Helder in November–December, when the cold of winter sets in, were exceptionally late in 1956. This was, no doubt, due to the mild weather. *Mustelus vulgaris* and *Octopus vulgaris* made their appearance later in December, and the main crowd of *Scylliorhinus canicula* and *Raia montagui* did not arrive until January, 1957, which is quite unusual.

It struck Mr. Kristensen in 1956 that the stranding of live specimens of oceanic species, e.g. the saury pike *(Scomberesox saurus)* and the bream *(Brama raii)*, seemed to have another cause than had hitherto been supposed. Mr. Verwey had always assumed that these species would strand alive because of their inability to determine the depth of water, which would prevent them from regaining deep water once they found themselves in the shallower water near the coast. Mr. Kristensen now thinks that stranding may to a great extent be brought about by low temperature and violent commotion of the water in stormy weather. This supposition (De Levende Natuur, **59**, p. 59–64, 1956) was partly derived from the behaviour of tropical fish, which will show desorientation if irritated by low temperature, partly from the fact that stranding often occurs during stormy weather. The data may indicate that coordination of sensory impressions is hampered by low temperatures.

A list of migrants and of some other specimens landed in 1956 is added to this report as usual. Special mention be made of a female electric ray *(Torpedo marmorata)* that was caught northwest of Terschelling in September. It is a southern species, which reaches the North Sea only by way of exception. Four specimens have now been reported in all; a short article on the subject appeared in De Levende Natuur, **60**, p. 39–41, 1957.

Mr. Kristensen also gave special attention to the silverside *(Atherina presbyter)* and to the occurrence of the Zuiderzee herring near Den Helder.

Hitherto little was known of the silverside in our country. Thanks to the help of Mr. G. J. de Haan, Texel, it was now ascertained that the species spawns in brackish water on the island of Texel in June and July. The fingerlings disappear when about 5 cm long, and return the next year when they are 7–13 cm long. Just as the anchovy, to which

the silverside greatly resembles in migration and distribution, the majority of the spawners are one year old, only a few are longer (about 16 cm) and two years old. On this species Mr. KRISTENSEN also published a short paper (De Levende Natuur, **60**, p. 159–162, 1957).

Herrings with the characteristics of the former Zuiderzeeherring gradually disappeared after the closure of the Zuiderzee, between 1932 and about 1940. In 1956 it appeared that such herrings were still caught in the brackish water of the North Holland Canal. In the months of April and May Mr. KRISTENSEN managed to get hold of some 20 specimens. According to the fishermen he bought them from they had all characteristics typical of the former Zuiderzee herring. This was confirmed by Mr. J. ZIJLSTRA, IJmuiden, who found that the numbers of vertebrae agreed with those of the Zuiderzee variety and not with those of the North Sea herring. The herrings in question probably belong to the populations of the Eems, the Jade or the Elbe, where spring spawning herrings maintained themselves in the brackish waters.

The grey seal *(Halichoerus grypus)* already mentioned in the previous report was repeatedly seen during the first half of the year 1956. Since the animal was by no means shy it may have come from the protected colony of the Farne Islands, some 400 km northwest of Den Helder. The observations were taken down by Mr. KRISTENSEN (De Levende Natuur, **60**, p. 62–63, 1957).

Mr. VERWEY gave much time to the interpretation of the literature on the vertical movements of plankton animals as a result of work done at Den Helder. He delivered a lecture on the subject at the meeting of the Society of April 21st. He also gave a survey of our present knowledge of the influence of temperature on the osmoregulation of crustaceans, which may be useful in designing future work. He read a paper on this subject during the Colloque internationale de biologie marine, held at Roscoff in June (Ann. biologiques, **33**, p. 129–149, 1957). Mr. VERWEY also gave an account of the organization of marine biological research (Rapporten Kon. Ned. Akad. van Wetenschappen, **8**, p. 1–16, 1956). Finally, mention should be made of an evaluation of the numbers of birds present in the Wadden area during the severe cold in the month of February (Ardea, **44**, p. 218–224, 1956). The estimates were made by Mr. KRISTENSEN and Mr. VERWEY from a plane that was placed at their disposal by the Royal Navy at the request of the Committee for Birdfeeding in Winter.

The hydrographic investigations, under the direction of Mr. POSTMA, had all the benefit of Mr. DUURSMA's appointment. On March 1st Mr. DUURSMA, a chemist in the service of the Netherlands Organization for Pure Research, started a study on the quantity of dissolved organic

matter in the sea. The amount of this material many times exceeds the quantity of organic matter present in plankton and organic detritus. Nevertheless, its concentration is so low that considerable difficulties are encountered if one tries to obtain accurate determinations. As a result only relatively few estimates have as yet been made.

The activities of Mr. DUURSMA were in the first place directed to the development of an accurate method of analysis. The principle of the original carbon-determination of KROGH, which was modified by KAY, is the oxidation of organic carbon to carbon dioxide, followed by a titration of the latter compound. Mr. DUURSMA has substituted this titration by the so-called coulometric determination, which is used for the determination of carbon in steel. It may already be stated that the latter method has many advantages to the method of titration. Besides carbon, the nitrogen-content of the dissolved matter will also be determined. It is to be expected that the C:N - ratio will give indications about the character of the material in question.

Mr. POSTMA continued his investigations on the influence of Wadden Sea water in the North Sea, already mentioned in the previous annual report. Continuing research work carried out at Den Helder in 1954 by Prof. J. KREY of Kiel University, Mr. POSTMA tried to obtain insight into the ratio of chlorophyll in living and dead phytoplankton. This investigation is a continuation of the work on productivity, which has been going on for a number of years. In this connection it was a fortunate circumstance that Prof. E. G. STEEMANN NIELSEN, Copenhagen, in August passed a week at Den Helder, where he carried out a number of measurements of organic productivity by means of his C^{14} method.

Mr. POSTMA also spent much time on working out the chemical data of the "Snellius"-expedition; in September he delivered a short lecture on the results of this investigation for an assembly of German geophysicists at Hamburg.

Mr. BEKE's work concerning the chemical composition of suspended non-organic matter in the Waddensea came to a temporary stop by the reconstruction of the chemical laboratory in the second half of 1956. However, Mr. BEKE spent much time in assisting Mr. DUURSMA. The work on suspended matter will be continued in the future.

In the 1955-report the work of Mr. EVERHARDUS, a pupil of Prof. GROEN, De Bilt, was mentioned. Mr. EVERHARDUS investigated the movement of tide-rips in the Wadden Sea. His results are partly a confirmation of facts previously found, partly they give us better insight into the formation and disappearance of these boundaries of water masses. However, to obtain a more satisfactory insight, these investigations should also be continued.

In addition to the above mentioned activities, Mr. I. J. HERMANS,

Amsterdam, worked for three months at Den Helder. His work there preceded his appointment as a biologist at the Caribbean Marine Biological Institute, Curaçao, and with a view to his future task he intended to get familiar with the cultivation of plankton and molluscs.

A satisfactory collaboration has been maintained with various public agencies, which either gave their help or called in the aid of the Zoological Station. They are not mentioned separately, except the Pilotage and its Commissioner at Den Helder and the crews of two of the lightships, to whom we owe many thanks for their cooperation.

The summer courses were delivered by all the members of the staff just as in previous years. The number of attending students amounted to 36, 15 of whom came from Groningen, 1 from Utrecht, 2 from Amsterdam and 19 from Leyden.

Moreover, a special physiological course was organized by the University of Amsterdam for 20 of its students under the direction of Messrs. PUNT, VAN NIEUWENHOVEN, KUCHLEIN and PARMA. Another course for 34 younger students from Utrecht was organized by Mr. KIPP and his assistants.

The total number of man-days for individual workers and summer course participants at the Zoological Station in 1956 exceeded 1200. In the period 1947–'56 it averages about 1120.

The following foreigners visited the Zoological Station in 1956: R. H. BAIRD, Conway, Wales; Theodore H. BULLOCK, Los Angeles, California; Robert S. DIETZ and wife, Office of Naval Research U. S. Navy, London; D. C. GEYSKES, Paramaribo, Surinam; F. VAN HARREVELD and wife, Pasadena, California; P. C. HENRIQUEZ, Willemstad, Curaçao; Arthur D. HASLER, Madison, Wisconsin; H. KLEEREKOPER and wife, Hamilton, Ontario (Canada); C. LAD PROSSER, Urbana, Illinois; E. STEEMANN NIELSEN and wife, Copenhagen, Denmark; C. A. G. WIERSMA and wife, Pasadena, California.

The building has again undergone various improvements. By the end of 1955 the Ministry of Education, Arts and Sciences set f 21 000.— apart for this purpose. This sum was used for a modernisation of the chemical laboratory, an extension of the seawater pipings in the course room, the construction of fixed tables in the course room and in one of the students' rooms, the building of cabinets for storing supply specimens and for the faunistic museum collection, and, finally, a colovinyl covering of the wooden floors. Moreover, a lightning-conductor was put up on the roof of the building, a cellar was made for a heavy compressor, and a bicycle shed was built.

Even the students' lodge "Potvis" was rather expensive this year, as the roof needed a new rubberoid covering.

A considerable extension was given to experimental equipment.

Grants from the Netherlands Organization for Pure Research made the construction of a pressure chamber and the experimental installation for the work of Mr. De Blok possible, the control mechanism for his experiments was completed, a heavy compressor and a motor unit were installed and plastic pipings were laid on. These acquisitions required about *f* 28 000. – from the Organization for Pure Research and some thousands from the normal budget.

Mr. Duursma's work was also rather expensive. The above mentioned apparatus for coulometric nitrogen determination cost about *f* 1 600.—, but in addition to this more than *f* 1 000. – was spent on the completion of this apparatus and on some glassware. Moreover, a Beckman pH-meter was bought at the price of *f* 1 675.—. Another *f* 1 500. - was contributed to the compressor, so that the budget for instruments showed a total sum of *f* 6 500. – . This amount could only be paid because there was still some money left from former payments by the "Shell"-concern and by "Waterstaat".

The library, especially the section for periodicals, is cramped for space, but the library itself is in an excellent condition. The available budget, which is low, was largely exceeded, and we hope that in future years this amount may be a little increased. A special sum enabled the purchase of a series of 50 volumes of the Swedish periodical "Fauna och Flora" and four volumes of the journal "Deep Sea Research". Other series were completed and the exchange programme was carried on and extended.

The skipper, J. H. Oversluizen, kept the Station's vessel "Max Weber" in good repair, but by the end of the year severe difficulties had to be faced. Upon reviewing the motor the naval inspector demanded replacement of the cylinders. When this had been done, the engine block began to show cracks, and as this type of engine is obsolete, the block had to be cast to order. So the ship could not be used from September 1956 till March 1957, and the costs of these repairs amounted to about *f* 5 500.—, which, we hope, will be refunded by the Government.

The Station's supply with seawater has been a matter of special concern for many years. Formerly, the water had been pumped up from the surface of the Nieuwediep during flood tide. When the south end of the Nieuwediep was dammed off in 1951 the harbour was no longer under the influence of tidal currents. From this time on the quality of the water in the harbour Nieuwediep became bad in summer and a water boat had to be used to transport water from the Marsdiep, the inlet of Texel. In winter the water of the harbour could still be used when pumped up from near the bottom. By 1956 the situation had grown worse by silting to such a degree that the Station had to rely

fully on transportation of water by boat. In this way excellent water, of a higher salinity than formerly, was provided.

There were some changes in the staff during the year 1956. In the first place there was the departure of Mr. KRISTENSEN, who joined the staff of the Physiological Laboratory of the University of Amsterdam on November 1st. From 1946 onward he had been at the Zoological Station and he had devoted himself to his task with great enthousiasm. The loss of his faunistical and ecological knowledge will be deeply felt for the next few years. Above all things he was always of great help with the courses, and he was very good in supplying the Universities with animals. Mr. W. H. DUDOK VAN HEEL from Utrecht was appointed in his place and entered upon his duties on November 15.

In June Mr. J. H. OVERSLUIZEN was given the combined job of skipper, motordriver and handiman. These functions cannot easily be combined and we hope that this situation will soon be altered. The former combination of caretaker and skipper was no longer kept up. At present Mr. BEKE, a chemical analyst, is living in the building. By means of this measure two garrets became vacant, which was a piece of good luck, as a shortage of space is gradually being felt.

Miss G. WATTIEN, a student-analyst, chose another career on September 1st. Her place was taken by Mr. THEO DE VRIES. Finally, we are sorry to state that the charwoman Mrs. D. FERNHOUT-GLAS left the Station on December 15th after having been in our service for more than six years.

Thanks to a grant given by the Netherlands Organization for Pure Research Mr. E. K. DUURSMA could commence his activities on March 1st.

It should be mentioned that Mr. POSTMA paid a short visit to Norway with the purpose of making investigations in the fjords under the auspices of the Shell-concern. On this occasion he visited Prof. BRATTSTRÖM at Bergen, Prof. BRAARUD at Oslo and Prof. KULLENBERG at Göteborg. In September Mr. POSTMA attended the conference of the Assembly of geophysicists at Hamburg.

Mr. VERWEY joined the Colloque internationale de biologie marine, which was held at Roscoff in June. His holidays in September were devoted to visits to the marine biological laboratories at Arcachon, Concarneau, Dinard and Luc-sur-Mer and the fisheries laboratories at La Tremblade, La Rochelle and Boulogne-sur-Mer, as well as to the laboratory of the Centre de Recherches et d'Etudes océanographiques at La Rochelle. This was undertaken to get an impression of the organization of marine biological research in France.

The supply of study materials amounted to *f* 5 911.70. The purchase of animal specimens cost *f* 2 954.59 and expenditure for preserving

solutions, wicker-bottles and packings was *f* 1 174.29. This resulted in a surplus of *f* 1 781.82, which exceeded the profit of previous years. The low sum for purchases of specimens was partly due to the fact that sharks were only landed in small numbers this year.

The expenses for 1956 were *f* 117 786.68. The estimated amount was *f* 114 300.—, so that there is a deficit of *f* 3 486.68. We hope the Government will decide to refund this deficit. Of the amount of *f* 117 786.68 *f* 93 790.26 was spent on salaries and national insurance and *f* 23 996.42 on the actual exploitation. In fact, expenses have been much greater because of the grants from the Organization for Pure Research. Moreover, the Government settles pension payments directly with the National Pension Fund. The total expenses may have exceeded *f* 150 000.—.

Den Helder, February 1957

J. Verwey

COMPTES RENDUS DE LA SOCIÉTÉ NÉERLANDAISE DE ZOOLOGIE

JANVIER 1955 – DÉCEMBRE 1956

Séance du 8 janvier 1955 à Amsterdam

1. L. F. DE BEAUFORT (Amsterdam). Les résultats des recherches sur la Zuiderzee - IJsselmeer de la Commission – Zuiderzee de la Société Néerlandaise de Zoologie.

Voir: Veranderingen in de Flora en Fauna van de Zuiderzee (thans IJsselmeer) na de afsluiting in 1932, rédaction: L. F. DE BEAUFORT; De Boer, Den Helder, 1954, 326–329.

2. K. H. VOOUS (Amsterdam). L'origine zoogéographique et l'évolution des oiseaux d'Aruba, de Curaçao et de Bonaire.

Voir: Acta XI Congr. internat. Ornithol., Basle, 1955.

3. W. G. WALTER (Leyde). Kinetics of pupillary dilation induced by administration of l-epinephrine.

Voir: Acta physiol. pharmacol. neerl., **3**, 1954, 309–324.

4. J. LEVER (Amsterdam). La fondation et les projets de recherches du laboratoire zoologique de l'Université Libre.

Un résumé de cette conférence n'a pas été remis au secrétaire.

Séance du 12 février 1955 à Amsterdam

J. J. A. VAN IERSEL (Leyde). L'analyse du comportement sexuel. Un résumé de cette conférence n'a pas été remis au secrétaire.

Séance du 8 mars 1955 à la Haye, organisée avec le concours du Cercle Chimique de la Haye et de la Société Royale "Diligentia".

1. J. A. KUYPER (Amsterdam). Les aspects chimiques de la perturbation de l'équilibre biologique dans la nature par l'application de substances phytopharmaceutiques.

2. D. J. KUENEN (Leyde). Les aspects biologiques du même problème.

Les résumés de cettes conférences n'ont pas été remis au secrétaire.

Séance du 19 mars 1955 à Utrecht

1. J. W. SLUITER (Utrecht), aussi de la part de P. F. VAN HEERDT et

J. J. Bezem. Recherches statistiques sur les populations de chauve-souris en Zuid-Limburg.

Voir: Arch. néerl. de Zool., **12,** 63–88, 1956.

2. J. de Wilde (Wageningen). La périodicité dans le réveil du sommeil hibernal chez les chauve-souris.

Voir: de Wilde et van Nieuwenhoven, Publicaties van het Natuurhistorisch Genootschap in Limburg, Série **7,** 1954.

3. P. J. van Nieuwenhoven (Amsterdam). Les changes dans la population de chauve-souris d'une grotte pendant la seconde moitié de l'hiver. Un résumé de cette conférence n'a pas été remis au secrétaire.

Séance du 30 avril 1955 à Utrecht

1. C. P. Raven (Utrecht). Sur trois types de mécanismes déterminatifs chez *Limnaea.*

Voir: Raven, de Boon et Stadhouders, Journ. embryol. and exp. morphol., **3,** 1955; Raven et Beenakkers, Journ. embryol. and exp. morphol., **3,** 1955.

2. M. W. Woerdeman (Amsterdam). On embryonic induction.

After explaining what must be understood by embryonic induction and after having given some examples (induction of neural plate by the underlying archenteric roof, induction of the lens by the optic vesicle), embryonic development is defined as a series of induction processes, some of them occurring synchronously, others in a certain regular succession.

In the development of many organs there is not one single inductor but the development of those organs is the result of the action of a group of inductors (coördinated inductor systems, according to Holtfreter).

It is not yet sufficiently known which inductors constitute such systems and how they coöperate. Perhaps some of them inhibit the others at a certain moment or reinforce their action. It seems to be certain, however, that an inductor may belong to more than one system of coördinated inductors.

Investigations on the nature of the induction factors (induction by killed inductors, extracts of inductors and tissues of adult animals) have drawn the attention to the role played during induction by the reacting cells. After explaining what is to be understood by the competence of the reacting system and after having demonstrated how this competence changes in the course of embryonic development, induction is shown to be dependent on the nature of the inductor(s) and the competence of the reacting system. The importance of the genetic properties, especially of the reacting system, is stressed.

The name "organizer" seems to be incorrect; the term "inductor" is preferred, because in many cases "organisation" is the result of the properties of the reacting system and much less of those of the inductor,

although examples can be cited where the inductor influences the organisation of the organ formed by the reacting system.

In answering the question: "what is induction?" several opinions are mentioned (activation, evocation against individuation, inductive substances, specificity of induction processes, etc., etc.).

Undoubtedly chemical factors are of great importance, although physical factors may not be neglected. There certainly is no single "induction substance" and induction may in one case be the result of processes completely different from those working in other cases.

The importance of further research (biochemistry of enzymes, specific proteins, and a serological approach to the problems of induction) is stressed.

3. P. D. NIEUWKOOP (Utrecht). L'analyse du processus d'induction neurale.

Voir: NIEUWKOOP *et al.*, J. exp. Zool., **120,** 1–108, 1952; NIEUWKOOP et VAN NIGTEVECHT, J. embryol. exp. morphol., **2,** 175–193, 1954; NIEUWKOOP *et al.*, Proc. Roy. Acad. Sci. Amsterdam, 1955.

4. H. A. L. TRAMPUSCH (Amsterdam). Sur la régénération. Un résumé de cette conférence n'a pas été remis au secrétaire.

5. G. TEN CATE (Amsterdam). Chemical embryology and "developmental mechanics".

A greater part of chemical embryology is purely descriptive, studying the chemical composition of the entire embryo and of its parts, dealing even with single cells and intracellular particles. The results from earlier work were collected by NEEDHAM (1931). Since then, substances like enzymes and other proteins, nucleic acids, vitamins, etc., received most attention. At the moment some information is available about the chemical diversification in the embryo which accompanies morphological differentiation. Furthermore, the study of embryonic metabolism in various periods of development and in various regions of the embryo has contributed to the knowledge about chemical processes occurring during fertilization, cleavage, gastrulation, etc. BRACHET's book (1947) contains many data of this kind. As an example I may mention that glycolysis was found to be important for the production of energy, needed for the movements occurring during gastrulation (WOERDEMAN, 1933; HEATLY and LINDAHL, 1935, 1937; JAEGER, 1945; COHEN, 1954).

The study of induction in particular brought chemical embryologists more closely into contact with developmental mechanics. This is expressed in the title of NEEDHAM's book of 1942: "Biochemistry and Morphogenesis". The fact that neural induction can be obtained by killed tissues, induced biochemists to seek for a chemical agent, sometimes indicated as an induction hormone. Although this line of research still is being continued, many biochemists were disappointed when it be-

came apparent that neural induction can be evoked by various kinds of substances, the specificity of the effect then residing (in these experiments) only in the reacting ectoderm which, even by simply damaging it through the effect of a nonphysiological pH of the culture medium, may be induced to organize a neural tube. Therefore it may be advantageous to reduce the baffling complexity of the problem of organ-formation by studying first the reacting ectoderm only.

The results of developmental mechanics have made it clear that the specific nature of morphological differentiation is already determined some time before its appearance.

This might be the consequence of some invisible, chemical differentiation, often called chemodifferentiation. In tentative explanations of the development of different organs in diverse parts of the embryo this chemodifferentiation has often been indicated to consist of local variations in pH, permeability, concentration of nucleic acids, enzymes, vitamins, etc.

However, it remains an enormous problem to elucidate the mechanism through which such chemical or physical diversification may direct the course of development towards one specific organ with the exclusion of other possibilities. The analysis of chemodifferentiation may be started with a study of the chemical differences between various completely differentiated organs in the adult state. It is known that certain quantitative differences exist in the concentration of ubiquitous enzymes, but it seems more attractive to search for qualitative, or at least very large quantitative differences between one organ and the others, as are found in some enzyme systems of the liver. Furthermore, there are substances which occur in one specific organ only (organ-specific substances), as could be shown by means of serological methods. In this way Ebert (1952) was able to find specific heart proteins in primitive streak stages of chicken embryos. In our laboratory a study is made of the development of the crystalline lens of the eye. In chicken and frog embryos a positive precipitin reaction showed the presence of lens-specific antigens in lens-pit and lens-bud stages respectively, and thus prior to morphological differentiation (Ten Cate and Van Doorenmaalen, 1950). This may be considered as evidence of chemodifferentiation, since other parts of the embryo did not contain demonstrable amounts of lens substances. Probably because of the larger size of his object of study, Ebert obtained positive results in much earlier stages and moreover found indications of a process of segregation, in that heart myosin first was found throughout the entire embryo, whereas in the head-process stage it was restricted to the presumptive heart region only (1954). One might assume that determination of this region depends on a monopoly with regard to these organ-specific

substances and (or) on the rise of their concentration above a certain threshold value.

We continued our work in two directions: firstly, by the application of a serological histochemical method, using fluorescein-labeled antiserum (COONS' technique), VAN DOORENMAALEN is studying the presence of lens antigens in microscopic sections. Secondly, we are engaged in a study of the properties of one protein component in particular, *viz.* α-crystallin, which seems to be suitable for our purpose of isolating even small quantities of organ-specific substance from a large amount of material in order to identify it by serological and other methods. In this way it can be investigated how big is the difference between the lens and other parts of the adult organism as regards the concentration of this protein. It may be expected that in those species where the iris is able to regenerate a lens, it will contain some lens protein; in other organs the quantity of this protein probably is very small if not undetectable. The quantitative growth of α-crystallin in a series of 4- to 14-day-old chicken embryos was assessed by ZUIDWEG (1954) by means of isoelectric precipitation of the crude fraction. He found that with increasing age the percentage of α-crystallin in the total lens protein decreases. In other words, this protein showed its highest relative concentration in the earliest stages and therefore it seems justified to search for it in this period of development, where it might be found perhaps even in the oocyte. Moreover, it may be studied whether lens induction is accompanied by a rise in quantity of this protein. WOERDEMAN (1950) found that, when presumptive lens ectoderm or optic vesicle are ground and after centrifugation the clear supernatant is incubated during 24 hrs. at 37°C. these extracts do not react with lens antiserum, but if they are combined and incubated for the same time, they gave, in a number of cases, a positive precipitin reaction.

When tracing the specific lens proteins backwards in development we encounter the problem of their origin. Do a small number of protein molecules occur already in the oocyte, prototypes, produced by genes in the germinal vesicle and released before or after maturation? Or do they originate *de novo* from a limited number of precursor molecules which undergo evolution in various directions, thus producing proteins of various types? Though somewhat more concrete, it is essentially the same question as that which ROUX posed, when he wrote about preformation and epigenesis of a number of "Qualitäten". The answer can only be given by (positive!) experimental results.

Personally I am inclined to think that most proteins originate from specific templates in the genetic apparatus (of nucleus or cytoplasm) and that only minor modifications remain possible by such adaptive phenomena as have been described to occur in the biosynthesis of

enzymes. Perhaps the local variations in pH, substrates, etc., might then provide different conditions by which one type of organ-specific substance could be produced more rapidly than another one, and thereby determine the course of morphological differentiation.

References:

BRACHET, J., 1947: Embryologie Chimique, Paris.
COHEN, A. I., 1954: Physiol. Zool,, **27,** 128.
EBERT, J. D., 1952: Ann. N. Y. Acad. Sci., **55,** 67.
EBERT, J. D., 1954: VIIIth Int. Congr. Cell Biol., Excerpta Medica, Sect. I, **8,** 390 (abstract).
HEATLEY, N. G., 1935: Biochem. J., **29,** 2568.
HEATLEY, N. G., and P. E. LINDAHL, 1937: Proc. roy. Soc. B, **122,** 395
JAEGER, L., 1945: J. cell. comp. Physiol., **25,** 97.
NEEDHAM, J., 1931: Chemical Embryology, Cambridge.
NEEDHAM, J., 1942: Biochemistry and Morphogenesis, Cambridge.
TEN CATE G., and W. J. VAN DOORENMAALEN, 1950: Proc. Kon. Ned. Akad. Wet. Amsterdam, **53,** 894.
WOERDEMAN, M. W., 1933: Proc. Kon. Ned. Akad. Wet. Amsterdam, **36,** 189, 423.
WOERDEMAN, M. W., 1950: Versl. Vergad. Kon. Ned. Akad. Wet. Amsterdam, **49,** 58.
ZUIDWEG, M. H. J., 1954: Proc. Kon. Ned. Akad. Wet. Amsterdam C, **57,** 115.

Séance du 21 mai 1955 à Leyde

1. D. A. HOOIJER (Leyde). Sur les vertébrés du Quaternaire de Célèbes.

Voir: Vakbl. v. Biol., **35,** 153–163, 1955.

2. G. C. JANSEN (Amsterdam). The behaviour of cattle and the "cafetaria"-stable.

Cattle raisers have in recent years introduced a new type of stable, the „cafetaria"-stable. In such stables the cattle move around freely but each animal has its own feeding site at which it is fed at fixed times every day. This ensures that most of the manure is collected in the drain. The behaviour of cattle in such a stable and the training required for proper functioning of the stable have been studied.

A social rank order is established within a few days and remains constant from then on, even in spite of environmental changes. A few cows which fell seriously ill during the observation period nevertheless kept their social status.

Among three cows, two different types of rank order may exist: (1) A is superior to both B and C, B is superior to C only, "resultant triangle", or (2) A is superior to B, B is superior to C, and C is superior to A, "cyclical triangle".

In the herd studied, which comprised 34 animals, 124 resultant triangles were found. This shows that this type of rank order is quite common. If the cattle are arranged in accordance with the established

rank order, the distribution of resultant triangles within the herd is found to be approximately normal.

A "coefficient of consistency" has been introduced which gives an impression of herd structure. In the herd studied the value of this coefficient was 0,92.

The social status of the individual is correlated with its age and the suitability of its horns for fights, the values of the correlation coefficients between status and these two factors being + 0,73 and + 0,50, respectively.

3. H. J. DE FLUITER (Wageningen). Leafhoppers as vectors of plant viruses.

Plant viruses can be transmitted from diseased plants to healthy ones in a mechanical or in a biological way. In the latter case the virus is transmitted by an organism, the vector. According to the persistence of the virus in the vector, the viruses are divided in two main groups, *viz.* the non-persistent and the persistent viruses. In the case of biological virus transmission an obligatory relationship between virus and vector is present. In many cases this relationship is indicated by a high degree of specificity.

The cicadellid leafhoppers constitute the larger group of vectors of biologically transmitted viruses. According to MARAMOROSCH almost all leafhopper borne plant viruses undergo an incubation period in their insect vectors.

Vectors of virus diseases are found among the following orders of insects:

THYSANOPTERA

Examples: *Frankliniella lycopersici* and *Thrips tabaci*. They are vectors of spotted wilt of tomato and of yellow spot of pineapple.

HEMIPTERA – HOMOPTERA

Aphididae. Aphids transmit the largest number of plant viruses. Many acquire the virus in short feeding periods and transmit it almost immediately; however, they lose the virus very soon. In some cases, however, (*e.g.*, potato leafroll, persistent strawberry viruses, a.o.) the aphids are unable to transmit the virus to healthy plants immediately. In this case an incubation period occurs between the acquisition of the virus and its transmission. After an incubation period (latent period), varying in length in the different cases, the vector becomes infective. The plant virus in this case is retained by the vector for a considerable length of time, often for the remainder of its life. In this case we speak of biological transmission.

Cicadellidae, *Fulgoridae*, *Aleyrodidae* and *Coccidae*. Many representatives of these groups are vectors of virus diseases. The cicadellid leafhoppers

constitute the largest group of vectors of biologically transmitted viruses. Aleyrodidae are well known vectors of virus diseases of tobacco and cotton in the tropics.

The swollen shoot disease of cocoa is an example of a virus disease transmitted by mealy bugs.

HEMIPTERA – HETEROPTERA

A few bugs are known as vectors of virus diseases. The most important one in the eastern part of Western Europe is *Piesma quadrata*, the vector of the "Kräuselkrankheit" of sugar beets.

COLEOPTERA

Diabiotica-species are known in the U.S.A. as vectors of the squash mosaic. The relationship between vector and virus is not yet fully investigated.

ACARINA

A few mites are able to transmit viruses. *Eriophyes ribis* Nal. is for example the vector of the reversion disease of black currant (MASSEE) and the mite *Aceria tulipae* was recently described as the vector of wheat-streak mosaic in Canada (SLIJKHUIS).

The relationship between the virus and the vector in leafhopper borne viruses is often highly specialised because of the fact that:

1. in many cases the virus shows a distinct vector-specificity; only one species or a few closely related species are able to transmit the virus notwithstanding the fact that the virus itself occurs in a great number of different host plants (example: the aster yellow-virus is found in more than 50 host plants; *Macrosteles fascifrons*, however, is the only vector);

2. almost all leafhopper borne viruses undergo an incubation period in their insect vectors;

3. most leafhopper borne viruses are persistent viruses;

4. plant virus multiplication in leafhopper vectors has been demonstrated by FUKUSHI (rice stunt), BLACK, KÜNKEL and MARAMOROSCH (aster-yellows), BLACK (club-leaf), MARAMOROSCH, BLACK and BRAKKE (wound-tumor), KÜNKEL and MARAMOROSCH (corn stunt);

5. in four cases transovarial passage of the virus from the mother insect to its progeny has been demonstrated;

6. leafhopper borne viruses are transmitted in a biological way. Mechanical transmission is not possible or very difficult;

7. leafhopper borne viruses are not seed or soil transmissable.

Plant viruses transmitted by leafhoppers are known from many parts of the world. The following examples can be mentioned:

U.S. America: Pierces disease of grapes, transmitted by 14 closely related species; curly top of sugar beet, transmitted by *Eutettix tenellus* Baker; aster-yellows transmitted by *Macrosteles fascifrons* Stal., peach yellows, transmitted by *Macropsis trimaculata* Fitch.

South America: curly top of sugar beet transmitted by *Agallia sticticollis* Stal.

Africa: streak disease of corn, transmitted by *Cicadulina mbila* China, *C. Zeae* China and *C. storeyi* China.

Japan: stunt disease of rice, transmitted by *Nephotettix apicalis* Motsch. var. *cincticeps* Uhl. and *Deltocephalus dorsalis* Motsch.

Australia and S.E. Asia: the Fiji disease of sugar cane, transmitted by *Perkinsiella vastatrix* Breddin and *P. saccharicida* Kirk.

It is remarkable that in Europe leafhopper borne viruses until recently were known only from the Russian Union, where the zakuklivania (= pupation disease or mosaic) of oats is widely distributed, occurring in the Far Eastern Province, Siberia, Central Kazakstan as well as in parts of European Russia. The disease is transmitted by *Calligypona (Liburnia) marginata* Fallen, a cicadellid which also occurs in Western Europe. Another leafhopper borne virus disease known from European Russia is the mosaic of winterwheat, which is transmitted by the Jassid *Psammotettix striatus* L.

From Western Europe until very recently no leafhopper borne viruses were known. In 1950 Prentice published a paper on a dwarfing disease of loganberry, blackberry, raspberry and other cultivated species and hybrids of *Rubus*. The disease being transmissable by grafting was considered to be a virus disease. In 1953 De Fluiter and Van der Meer showed by transmission experiments that this virus in the field is transmitted by the leafhopper *Macropsis fuscula* Zett.

In 1953 Maramorosch published a paper on a second leafhopper borne virus disease from Western Europe, *viz.* a previously unknown clover disease. According to Maramorosch the disease is a virus disease, which is transmitted by the leafhopper *Euscelis plebejus* ssp. *plebejus* (Fall.).

The investigation on Rubus stunt as a leafhopper borne virus disease carried out by De Fluiter and Van der Meer, in the Netherlands, are discussed.

A brief summary of the results obtained follows here.

Rubus stunt is a virus disease described by Prentice in 1950.

The first record of the disease on blackberry was published in Great Britain by Wormald and Harris (1932). The disease probably affects all *Rubus* species. Infection in all hosts first results in the production of numerous weak, short and thin canes, which gives the stunted plant a bushy appearance; later the flowers show typical proliferations; the

floral parts with exception of the stamens becoming foliar. PRENTICE called the virus causing this disease, Rubus stunt virus.

Since 1945 this disease has become increasingly prevalent in the Netherlands on raspberries in the production area near Breda, where it seriously interfered with the commercial cultivation of raspberries. The disease is also common throughout the Netherlands on wild blackberries (DE FLUITER and THUNG, 1951). There is, however, no correlation between the occurrence of the disease on wild blackberries and on cultivated raspberries. On one occasion it was also found on wild raspberries. According to RIETSEMA the disease has been present in the Breda area for 25–30 years; since 1945, however, there has been a sudden and rapid increase. In 1950 the authors were asked to investigate the control of the disease. Field observations indicated that the disease was vector borne. Transmission experiments showed that the disease is not aphid borne. Experiments made in 1952 showed, however, that the leafhopper *Macropsis fuscula* Zett. is a vector of the virus (DE FLUITER and VAN DER MEER, 1953).

The first symptoms on newly planted healthy canes appear in the field in the second year after the crop is harvested. Field experiments showed that infection of the plants with virus takes place in the months July, August and September when the adult leafhoppers spread the virus from diseased plants to healthy ones.

The biology of *Macropsis fuscula* was studied. The leafhopper hibernates as an egg, the larvae hatch in the second half of May, the first mature insects appear at the end of June or the beginning of July. Their number increases gradually. In the raspberry plantations in the infested area adult specimens are numerous in the second half of July, in August and the beginning of September. At this time the virus is rapidly spread from diseased plants to healthy ones. This was confirmed in field experiments. The relations between vector and virus are being studied. The virus seems to be a persistent one.

Rubus stunt disease can be controlled by:

a. destroying the virus. THUNG (1952) showed that the virus in diseased root stocks is destroyed by a heat treatment of 1 ½–2 hours in water of 45°C. In this way virus free plant material can be obtained (another way to obtain virus free material is to grow the plants from seed and raise them in isolated fields in areas where the disease is not present in blackberries or raspberries);

b. the control of the vector. Laboratory as well as field experiments showed that the overwintering leafhopper eggs can be killed by thouroughly spraying the canes in January or February with tar oil 6 %, DNC (AAnitro) 0.4 % or DNC (Trifocide) 0.25 %. Young larvae can be killed by a spring spray with parathion, a.i. 25 %, 0.1 %. Field

experiments showed that spraying first year plants with parathion 0.1 % every fortnight, starting on May 1st and continuing to October 15th, reduced the occurrence of Rubus stunt in these plots to a great extent. DDT had no effect.

The rapid spread of the disease since the end of world war II can be explained as follows. Until 1945 *Lampronia rubiella* Bjerk was controlled in the Breda area by a tar oil dormant spray. After 1945 DDT was introduced. The growers dropped the tar oil dormant spray and instead of it introduced a DDT spring spray to control *Lampronia rubiella* as well as *Byturus tomentosus* and *B. fumatus*. Since *Macropsis fuscula* is not sensitive to DDT, it developed into a pest; a small virus source was present. These two factors, *viz.* the presence of a virus source and the increase of the vector population caused the rapid increase of the Rubus stunt disease. Spread of apparently healthy but in fact already diseased plant material (incubation period of one year) also increased the spread of the disease over the Breda area.

In transmission experiments leafhoppers (nymphs as well as adults of *M. fuscula*) from blackberry were transferred to raspberries, and leafhoppers from raspberries were transferred to blackberries. However in all our experiments the transferred leafhoppers soon died. We now are inclined to believe there are two strains of *M. fuscula* or two different species of closely related leafhoppers, one living on raspberries and the other living on blackberries. The strain or species of raspberry will not breed on blackberry, while the strain or species of blackberry will not breed on raspberry. This gives an explanation for the fact that in the Netherlands in the field no correlation could be found between the occurrence of Rubus stunt on the wild blackberry and the cultivated raspberry.

References:

BLACK, L. M., 1941: Phytopathology, **31,** 120–135.
BLACK, L. M., 1950: Nature, **166,** 852–853.
BLACK, L. M., 1953: Ann. N. Y. Acad. Sci., **56,** 398–413.
BLACK, L. M., 1953: Phytopathology, **43,** 9–10.
BLACK, L. M. and M. K. BRAKKE, 1952: Phytopathology, **42,** 269–273.
FLUITER, H. J. DE and T. H. THUNG, 1951: Tijdschr. o. Plantenz., **57,** 108–114.
FLUITER, H. J. DE and F. A. V.D. MEER, 1953: Tijdschr. o. Plantenz., **59,** 195–197.
FLUITER, H. J. DE, H. H. EVENHUIS and F. A. V.D. MEER, 1954: Proc. Sec. Conf. on Potato virus Diseases, Lisse-Wageningen, 25–29 June, 1954, 84–88.
FUKUSHI, T., 1933: Proc. Imp. Acad. (Japan), **9,** 457–460.
FUKUSHI, T., 1940: J. Fac. Agr. Hokkaido Imp. Un., **45,** 83–154.
KUNKEL, L. O., 1937: Am. J. Botany, **24,** 316–327.
KUNKEL, L. O., 1948: Arch. Ges. Naturforsch., **4,** 24–26.
MARAMOROSCH, K., 1950: Phytopathology, **40,** 1071–1093.
MARAMOROSCH, K., 1952: Phytopathology, **42,** 663–668.
MARAMOROSCH, K., 1952: Phytopathology, **42,** 59–64.

MARAMOROSCH, K., 1952: Nature, **169**, 194–195.
MARAMOROSCH, K., 1953: vith congr. Internat. Microbiol. Rome, Sept. 6–12, 2, 163–166.
MARAMOROSCH, K., 1953: Plant disease Rep., **37**, 612–613.
PRENTICE, I. W., 1950: Journ. Hort. Sc., **26**, 35–42.
SLIJKHUIS, J. T., 1953: Phytopathology, **43**, 484–485.
THUNG, T. H., 1953: Tijdschr. o. Plantenz., **58**, 255–259.
WORMALD, H. and R. V. HARRIS, 1932: Rep. E. Malling Res. Sta., **49**.

Séance du 2 novembre 1955 à Amsterdam

1. S. DIJKGRAAF (Utrecht). Mouvements compensatoires des yeux et orientation chez les Crustacés.

Voir: Experientia, **11**, 407–409, 1955; Z. vergl. Physiol., **38**, 1956; Publ. Staz. Zool. Napoli, vol. jubil. Reinhard Dohrn, 1956.

2. C. J. VAN DER KLAAUW (Leyde). Problèmes de la surface crânienne.

Un résumé de cette conférence n'a pas été remis au secrétaire.

3. P. DULLEMEYER (Leyde). Architecture et structure du crâne de la vipère en rapport avec ses fonctions.

Un résumé de cette conférence n'a pas été remis au secrétaire.

4. A. PUNT (Amsterdam). Sur la respiration des insectes.

Un résumé de cette conférence n'a pas été remis au secrétaire.

5. J. J. FRIESWIJK (Amsterdam). The leech-defence reaction of *Physa fontinalis* (L.) and *Physa acuta* Drap.

When the mantle-lobes of *Physa fontinalis* are touched with a leech belonging to the genus *Glossiphonia* or with some slime of such a leech, a curious reaction can be observed. The snail strenuously lashes with the shell and at the same time it loosens the foot from the ground. Thus the animal retires with jumps out of the neighbourhood of the leech (DEGNER, 1921).

The reactions of *Physa fontinalis* and *Physa acuta* on being touched with *Glossiphonia complanata* (L.) do not show any marked differences. The leech-defence reaction is not only elicited by some species of leeches, but it is also possible to evoke the reaction by chemical stimuli, *viz.* by solutions of salts, among others NaCl and NH_4Cl in concentrations from 1 to 10% (WREDE, 1927).

My own experiments showed that *Physa fontinalis* is more sensitive than *Physa acuta* both to mechanical stimulation and to contact with *Glossiphonia complanata*. Various other species of leeches (*Helobdella stagnalis* (L.), *Hemiclepsis marginata* (O.F.M.), *Hirudo medicinalis* (L.)) appeared to be able to evoke the leech-defence reaction.

The mantle-lobes of *Physa fontinalis* were put into contact with solutions of different salts in comparable concentrations. It was proved that in NaCl and NH_4Cl the positive ion is responsible for the appearance of the leech-defence reaction.

By boiling extracts of minced specimens of *Glossiphonia complanata* it was possible to prepare a protein-precipitate, which elicited the typical leech-defence reaction on application to the mantle-lobes of *Physa fontinalis*.

The same reaction was caused by precipitates from extracts which were freed from free ions by dialysis.

Precipitates from extracts which, by adding acids or bases, were freed from protein-bound positive or negative ions, respectively, were also able to induce the leech-defence reaction. So it is evident that the reaction is caused by proteins and not by ions.

Intact specimens of *Glossiphonia complanata*, stored either in acid or in alkaline fluids, always caused the leech-defence reaction. This result is in accordance with the result of the experiments with extracts.

It seems possible that proteinlike materials, present in the slime of certain species of leeches, cause the leech-defence reaction of *Physa fontinalis*.

References:

DEGNER, E., 1921: Über einen Abwehrreflex bei *Physa fontinalis* L. Arch. Moll. K., **53**, 117–120.

WREDE, W. L., 1927: Über die Abwehrreaktion von *Physa*. Zoöl. Jahrb. (Zoöl.), **44**, 315–322.

6. V. S. VAN DER GOOT (Amsterdam). The utilisation of atmospheric oxygen by the tracheal system of *Locusta migratorioides*.

The utilisation of atmospheric oxygen by the tracheal system is defined as the percentage of the total amount of air oxygen, absorbed by the tracheal system in the course of normal breathing.

In order to measure the utilization, it is necessary to obtain pure expirational air. In general, sampling of tracheal air is difficult to achieve, because it is only possible to obtain air from a limited part of the tracheal system. KROGH applied this method of sampling in order to get an impression of the performance of the tracheal system. The respiration of *Locusta* is of an unusual type: the air enters through the foremost four pairs of spiracles, and leaves the body through the hindmost six pairs. This peculiarity is made use of in an experimental device whereby, with the aid of a diaferometer, the percentages of CO_2 and O_2 in expirational air of *Locusta* are measured continuously. In this way it is possible to measure the utilization of air oxygen in inactive and activated animals.

Séance du 7 janvier 1956 à Leyde

1. F. J. VERHEYEN (Utrecht). La réaction des animaux envers les lumières artificielles dans la nuit.

2. J. W. KUIPER (Groningue). Les organes de la ligne latérale comme récepteurs de vibrations mécaniques.

3. E. J. Slijper (Amsterdam). La naissance chez les Cétacées et chez d'autres mammifères aquatiques.

4. Mlle. J. Vis (Amsterdam). L'insertion des muscles au squelette.

5. Mlle. N. Croin Michielsen (Leyde). Recherches écologiques sur les musaraignes dans la nature.

Les résumés de cettes conférences n'ont pas été remis au secrétaire.

Séance du 18 février 1956 à Amsterdam

P. J. Gaillard (Leyde). La physiologie des glandes parathyroides.

Un résumé de cette conférence n'a pas été remis au secrétaire.

Séance du 17 mars 1956 à Utrecht

1. C. Romijn (Utrecht). L'équilibre calorique chez l'embryon d'oiseau.

Voir: Romijn et Lokhorst, Poultry Sci., **35,** 829–834, 1956.

2. W. Companjen (Wageningen). La régulation endocrine de la croissance et des fonctions sexuelles chez les sauterelles.

Un résumé de cette conférence n'a pas été remis au secrétaire.

3. P. Dullemeijer (Leyde). The shape of the brain in relation to the skull structure in a number of birds.

Voir: Dullemeijer et Tulner, Proc. Kon. Ned. Akad. Wet. C, **60,** 255–278, 1957.

4. J. J. M. L. Crombach (Leyde). La régulation de la sécrétion du suc gastrique chez *Rana esculenta* L. et *Rana temporaria* L.

Chez les mammifères, les oiseaux et probablement aussi les reptiles le cerveau et la moelle épinière remplissent une fonction non seulement dans la recherche de la nourriture mais encore, du moins partiellement, dans la régulation de la digestion. La sécrétion des sucs digestives est mise en action par des réflexes dans lesquels le nerf vagus et le nerf splanchnique du système nerveux sympathique jouent un rôle (Babkin, 1928).

Chez les amphibies et les poissons de tels réflexes ne se produisent pas (Wolvekamp et Tinbergen, 1942, et les recherches de Quispel à Leyde, (non-publiées)). Cependant on peut constater chez ces animaux-ci une sensible augmentation de sécrétion de suc gastrique après une excitation mécanique de l'estomac (cf. Friedman, 1937; Smirnoff, 1922).

D'une série d'expériences faites avec *Rana esculenta* L. et *Rana temporaria* L. il résulte que ce stimulus mécanique ne s'effectue pas sur la muqueuse, mais qu'il consiste dans l'extension de la paroi gastrique.

Après coupage des rami communicantes 5, 6, 7 et 8 et du tronc sympathique entre les ganglions 4 et 5 et 8 et 9 la sécrétion de pepsine causée par un stimulus mécanique, ne se trouve pas être interrompue. Le coupage du nerf vagus n'empêche pas cette sécrétion non plus.

Même quand les deux opérations sont effectuées sur le même animal, ce stimulus mécanique est encore effectif.

La conclusion générale qui se dégage de nos expériences est la suivante: Chez les animaux examinés et probablement aussi chez tous les amphibies et les poissons le cerveau et la moelle épinière ne jouent pas un rôle dans la sécrétion de pepsine. La régulation de la digestion chez ces animaux s'effectue donc plutôt dans la périphérie. S'il y a ici un réflexe, ce qui est vraisemblable, celui-ci doit se produire au moyen des ganglions sympathiques 5, 6, 7 et 8 ou du plexus d'Auerbach.

L'auteur veut exprimer toute sa reconnaissance à Mademoiselle M. I. C. P. DE JONG pour sa collaboration aux opérations.

Bibliographie:

BABKIN, B. P., 1928: Die äussere Sekretion der Verdauungsdrüsen. 2te Aufl. Berlin.
FRIEDMAN, M. H., 1937: J. cell. and comp. Physiol., **10,** 37.
SMIRNOFF, A. J., 1922: D'après un résumé dans: Ber. ges. Physiol., **13,** 87.
WOLVEKAMP, H. P. et L. TINBERGEN, 1942: Arch. Néerl. Physiol., **26,** 435.

5. H. P. WOLVEKAMP (Leyde). Quelques remarques sur l'osmorégulation par rapport aux caractères du système de circulation sanguine.
Voir: Acta physiol. et pharmacol. neerl., **4,** 1955.

Séance du 21 avril 1956 à Leyde

1. J. VERWEY (Den Helder), aussi de la part de L. J. ANTHEUNISSE et A. A. ALKHOLY. La migration verticale quotidienne du zooplankton.

Un résumé de cette conférence n'a pas été remis au secrétaire.

2. L. VLIJM (Amsterdam). The nature of the anti-thyroidal effect of Amphenone "B" in cockerels, studied by means of radioactive iodine.
Voir: Acta Endocrinol., **22,** 273–278, 1956.

3. J. METUZALS (Groningue). Les hémorrhagies intraparenchymateuses dans l'adénohypophyse comme mécanisme de l'effusion acute des hormones dans le sang.

Un résumé de cette conférence n'a pas été remis au secrétaire.

4. J. P. KRUIJT (Groningue). Speckling of the Herring Gull egg in relation to brooding behaviour.

Since 1950, under supervision of Prof. Dr. G. P. BAERENDS, fieldwork on the Herring Gull is being carried out on the island Terschelling. The aim is to find out which properties of the egg release the activity of retrieving an egg lying outside the nest. The experiments reported here are only concerned with the part played by speckling of the egg. The work is still in progress.

Method: The gull's own eggs are taken out of the nest and are replaced by a "normal" wooden model, which is visually similar to a natural gull's egg. In addition, two models, which differ in the way they are

speckled are placed on the rim of the nest. The experimenter, sitting in a hide at a distance of 5–10 meter from the nest, observes the behaviour of the gull when it comes back. One or both eggs may be rolled into the nest. The model that is rolled first is considered to have the highest releasing value. Each choice is offered many times; the results are evaluated statistically. In each subsequent experiment the type and the place of the models are changed to avoid conditioning to a special model or to a special place.

Results of the retrieving experiments: A speckled model is preferred to a plain model. Further experiments prove that (apart from a possible influence of the higher total darkness and of the colour of the speckles), the quality of "speckledness" as such increases the relasing value.

Secondly it is probable that at least two or possibly all of the following characteristics are favourable: the contrast of the speckles with the groundcolour must be high; the groundcolour itself must be light; the speckles must be dark and also darker than the groundcolour.

Lastly it was found that increase in number and reduction in size of the speckles heighten the releasing value, while total speckle-surface is of no importance.

On the basis of these findings a "supernormal" model was constructed. This possesses very many small, dark speckles on a light background. It appears that this model is preferred to a normal wooden model, however not to a natural gull's egg. Therefore still more detailed properties must be of importance.

Thus many properties of the egg determine the releasing value. It is not yet possible to say how many of these rest on an inborn basis and how many are learned. Work on this problem is in progress.

The importance of speckling for brooding success: The importance of speckling for the egg as a brooding object was also studied. In a number of nests the clutches were exchanged for plain-coloured models; in a control-series for speckled models. After two weeks most of the speckled clutches are normally incubated, while most of the plain-coloured clutches are destroyed. This result can be explained in two ways:

1. It could be that the plain models have so low a releasing value as brooding objects, that the owners themselves eat the eggs, or guard them less closely, thereby giving robbing gulls a better chance to destroy the nest.

2. It could also be that the plain-coloured clutches are more easily detected by robbing gulls.

Direct observation does not give a solution. The question was therefore tackled in a separate experiment.

Half of a number of artificial nests outside the colony were provided with speckled eggs, while the other half contained plain-coloured eggs.

The eggs used in this experiment were real gull's eggs, painted over in the desired colour. By direct observation of the robbing gulls and by checking the nests' content every half hour, it appeared that both kinds of eggs were robbed at the same rate. Therefore, although it is quite probable that speckling is a protective coloration against other enemies, it does not seem to be effective against Herring Gulls themselves.

This result justifies the conclusion that the first explanation of the first experiment is true. Thus plain-coloured eggs are inferior in releasing value as compared with speckled eggs, not only for egg-retrieving but also for brooding itself.

This conclusion has implications. Regularly gulls are found with plain-coloured clutches. These gulls also prefer speckled to plain models in retrieving experiments (which is evidence for, though not proof of the inborn basis of this preference). There are indications that the property of laying unspeckled eggs is hereditary. If so, then our experiments would lead us to expect that this property is selected against. In that case, the more careful guarding of speckled eggs will lead to an evolution of eggs with increasing differentiation of speckling.

5. P. D. Nieuwkoop (Utrecht). The necessity and possibilities of a quantitative analysis in developmental morphology, illustrated on the analysis of neural induction.

The developmental morphology has made great progress since the classical experiments of W. Roux, H. Spemann and others, and has given us a general insight in a number of processes which seem to play an important role in the development of the vertebrate embryo. Our knowledge is however still very fragmentary and in most cases only of a *qualitative* character. In order to bridge the wide gap between the morphological analysis on the biological level and the biochemical and physiological studies on the molecular level a quantitative analysis of the developmental processes seems to be quite essential. On the basis of the analysis of the neural induction some first steps towards a more accurate quantitative analysis are described.

The fold implantation technique (Nieuwkoop, 1952) and the sandwich technique (Nieuwkoop and v. Nigtevecht, 1954 and Sala, 1955) are critically discussed and as a result the spatial distribution of two principles (activation and transformation) playing a role in the regional induction of the central nervous system, is given in a diagram. Moreover, the competence of the ectoderm for neural induction, which has also been analysed with the fold implantation technique, could be studied quantitatively showing that in *Amblystoma mexicanum* the competence of the ectoderm for activation disappears already at middle to late yolk plug stage, whereas that for transformation probably lasts (considerably) longer.

The examples demonstrate that the fold implantation and the sandwich techniques can actually be used for quantitative analysis, although ameliorations in the existing techniques are highly desirable.

Séance du 8 septembre 1956 à IJmuiden

1. J. VAN MAMEREN (IJmuiden). Quelques développements récents dans la technologie de la pêche.

Un résumé de cette conférence n'a pas été remis au secrétaire.

2. R. TH. ROSKAM (IJmuiden). La conservation de poissons par rapport à la salubrité publique.

Un résumé de cette conférence n'a pas été remis au secrétaire.

3. C. L. DEELDER (IJmuiden). Recherches sur la migration de la montée *(Anguilla vulgaris)*.

Voir: Driemaand. Bericht Zuiderzeewerken, **35**, 117-119, 1954.

4. M. ROESSINGH (IJmuiden). Sampling problems in fish population studies in the sea.

At present the study of fish *populations* is the main object in fisheries science. The following characteristics must be known to understand the dynamics of a fish population: egg production, recruitment (number of young fish entering the exploited phase), growth, mortality and fluctuations. From these the most important is mortality, both natural and fishing mortality, because of its relation to the fishing yield. Now mortality must be deduced from density and age composition of the fish stock. A complete census of a fish stock is impossible. Therefore adequate sampling of the population is essential.

Samples can be collected with a research vessel or from the landings of the commercial fishing boats. The advantages of a research vessel are 1. the possibility of sampling at random over a big area, independent of the concentration of the fishing fleet and 2. the use of standard gear and standard methods of fishing. For special work, e.g. sampling of fish eggs, a research vessel is indispensable. A disadvantage is the restricted number of samples obtained by a single vessel and the expensiveness of the samples, especially when samples from an unexplored area are required (damage to gear). The sampling of herring, pilchard, etc. which are mostly concentrated in strongly marked schools, takes place with the aid of the echosounder: 1. the number of fish traces on the echogram is a rough measure of the density of the fish, and 2. generally spoken the samples are taken only when the echo sounder indicates the presence of fish.

Unlimited numbers of samples can be obtained from the market, but the fishing fleet is always searching for places where big fishes are concentrated, or the fishermen are fishing on traditional fishing grounds in certain seasons; therefore market samples are usually biased. At

present about 300,000 fishes are measured yearly on Dutch markets for research purposes, (herring, whiting, sole).

Age composition of Faroe haddock samples taken by a research vessel and from the market of Aberdeen trawlers:

Age	*R.V.*	*Trawler*
3	100	100
4	24	59
5	14	23
6 +	6	15

Further the demand of the public for special fish species, and the most abundant fish in a certain area play a part; e.g. fishing in the southern North Sea is mainly directed at the catch of flatfish and thus the gear is adjusted to flatfish.

Total catches from 7 pairs of hauls by an English and a Scottish research vessel, fishing together and each using the gear typical of their home ports.

species	*Scotland*	*England*
whiting	547	28
haddock	1147	547
plaice	24	56
rays	19	232

So the market does not give comparable data about the density and the age composition of all species. Alterations of gear, motor power, tonnage and number of vessels should be considered; therefore all samples must be converted with respect to a standardvessel.

Norwegian research was hampered by the discovery of a difference in catching power of different gears. Up to 1950 analysis of the stock of cod was based on samples from the catch of long lines and gillnets. Cod from the nets was larger, and the sex ratio was different from the cod caught with lines. But again higher numbers of large fish were taken after '50 when the purse-seine gear was introduced in Lofoten. Probably the purse-seine does not operate in a selective way. Thus all previous sampling from lines and nets has given biased length and age distribution of the population, and the mortality rates had to be re-calculated. The behaviour of the fish with respect to the net comes in the picture here, and it is expected that the study of fish behaviour will play a far more important part in the future.

Weather conditions also affect the catch; e.g. northerly winds often mean small catches in the North Sea. Weather conditions should be constant when sampling a certain area with a research vessel and thus the cruise programme should be carried out quickly before the weather changes. Therefore sampling continues day and night. But the be-

haviour of the fish is different during day and night, and also from place to place. In addition the fish reacts to the periods of the tide. To study the reactions of the fish to these changing factors, many short hauls during 24 hours or more at one position are necessary, supposing that the composition of the population does not change during the experiment.

Most species are caught in greater numbers during the night. Probably most fishes escape if the net is visible during daytime. Bottom fish sometimes is readily caught during the time it is feeding, e.g. at the beginning of the night. On the other hand some species move to levels above the reach of the bottom trawl during the night. Examination of the course of the catches during 24 hours may unravel this complex problem.

The duration of a haul with a trawl influences the catch as well. Fish accumulates in front of the net during the haul; hence it was expected that a haul of two hours would yield more than twice the number of fish than a haul of one hour. The opposite is true. Probably the hunted fish chase away the fish, which swim further forward, so that the percentage escaping increases during the haul.

Very different are the sampling problems in plankton work. Only one aspect is considered here. *Patchiness* of plankton organisms appears in different forms: 1. patches of several square miles are readily detected with a continuous plankton recorder or by sampling a grid of stations which lie some miles apart; 2. micropatches (centimeters – meters) are described from samples taken vertically at very short intervals; 3. patchiness also is present when samples are taken 100 m apart. To test the reliability of a sampling method for plaice eggs a series of hauls was made from bottom to surface with a standard plankton net.

The correlation between egg patches, salinity and temperature was slightly significant. Concluded was that two hauls are independent when they lie at least 25 m apart in the case of sampling young plaice eggs.

A striking fact is that the ratio of the different stages of development of the eggs was different at several stations close together: the more newly spawned eggs the less fully developed eggs, and v.v. This inverse correlation is not yet explained.

References:

Barnes, H. and S. M. Marshall, 1951: J. Mar. Biol. Ass., **30,** 233.
Hardy, A. C., 1944: Hull Bull. Mar. Ecol., **1,** 1.
Problems and methods of Sampling Fish Populations, 1956: Rapp. Proc. Verb., **140,** I.

5. L. K. Boerema (IJmuiden). Le cours de la population de plies près de la côte des Pays Bas après 1945.

Un résumé de cette conférence n'a pas été remis au secrétaire.

6. J. J. Zijlstra (IJmuiden). Population problems in the herring.
Voir: Rapp. et Proc. Verb. Cons. Int. Expl. Mer, in the press.

Séance du 3 novembre 1956 à Harlem

1. S. Dijkgraaf (Utrecht). Structure et fonction des statocystes des crabes.
Voir: Experientia, **11,** 407–409, 1955; **12,** 394–396, 1956.

2. Mlle. B. Baggerman (Groningue). La périodicité de la reproduction et de la migration chez l'épinoche.
Voir: Arch. néerl. de zool., **12,** 105–317, 1957.

3. P. G. W. J. van Oordt (Utrecht). The influence of environmental factors on the spermatogenetic cycle in the common frog *(Rana temporaria)*.
Voir: Van Oordt, thesis Utrecht 1956; Acta endocrinol., 1956.

4. C. O. van Regteren Altena (Leyde). Le cabinet paléontologique du musée Teyler à Harlem dans le passé et au présent.
Voir: Vakbl. v. Biol., **37,** 149–156, 1957.

Séance du 8 décembre 1956 à Amsterdam

1. W. L. M. Geilenkirchen (Utrecht). The use of Cartesian divers in cytophysiological research.

Un résume de cette conférence n'a pas été remis au secrétaire.

2. E. D. Nijenhuis (Utrecht). Quelques applications de la technique autoradiographique dans l'embryologie.

Un résumé de cette conférence n'a pas été remis au secrétaire.

3. L. de Ruiter (Groningue). Natural selection in *Cepaea nemoralis*.

Quantitative data on the intensity of natural selection in free-living animal populations are of crucial importance for current theories on the causes of evolution. Rough estimates of selection intensity may be based on observations on changes in allele frequencies alone. However, apart from selection other factors, such as interpopulation gene flow and genetic drift, may contribute to such changes. Moreover, overall selection at a given locus will often be the resultant of a host of conflicting ecological agents. For these reasons we can gain real insight into the selective process only if we try to specify the individual items on the balance sheet of allele frequencies.

The snail *Cepaea nemoralis* is very suitable for such work. Its shell colour and banding pattern are highly variable. The genetic basis of this variability is fairly well known. Almost all colonies are highly

polymorphic for the genes involved, and the relative frequencies of their alleles differ strongly from colony to colony.

Widely divergent views have been held as to the relative importance of selection and chance fluctuations as determinants of the genetic composition of any colony (e.g., DIVER, 1940; BOETTGER, 1931).

Circumstantial evidence for the effectiveness of selection has been given by CAIN and SHEPPARD (1954, and elsewhere) who noted a correlation between allele frequencies in different colonies and the colour and general appearance of their environment. Differential elimination of snails of different genotypes by predators hunting by sight is an obvious explanation of such correlations. Our data confirm the observation of CAIN and SHEPPARD that at least one of the snails' predators, the thrush, may exert such differential predation. In a colony at Haren, for instance, in which 27.5 % of the shells were yellow we found 42 % yellow shells among the thrush victims (M. H. A. KEENLEYSIDE and L. DE RUITER, unpublished).

CAIN and SHEPPARD have pointed out that differential predation is not a simple constant agent, but that the intensity and even the direction of selection by thrushes may change cyclically in the course of the seasons. Further, the intensity of selection by predators on a given gene also depends on the rest of the gene complex: banding may prove beneficial in combination with one, but harmful in combination with another shell colour.

The widespread occurrence of pleiotropy leads one to expect that many different environmental factors will contribute to the total selection acting on a given gene. Several facts support this view. The impression, based on field work, that yellow unbanded shells have a relatively high resistance to irradiation is confirmed by laboratory experiments (BOETTGER, 1954).

Some preliminary data obtained by us suggest that there may also be considerable differences in fecundity among the genotypes. Kept in captivity under conditions as nearly natural as possible, five-banded individuals appear to lay on the average almost twice as many eggs per year as do unbanded specimens and those with only the third band (37 as opposed to 22 and 14, respectively). On the other hand, no differences in fecundity were observed between snails with pink and yellow shells (Mrs. G. BALLINTIJN-DE VRIES and L. DE RUITER, unpublished).

In view of these facts the statement seems justified that selection is a powerful determinant of allele frequencies in *Cepaea* colonies, and that there may be a fierce tug of war between groups of environmental factors acting on the same locus in different directions. The resultant net selection will differ among colonies in accordance with differences

– perhaps seemingly slight ones – in their environments. This provides at least a partial explanation of the differences in genetic composition among colonies.

However, it does not explain the stability of the polymorphism in individual colonies. It is practically certain that gene flow between colonies does little to prevent selection from making colonies nearly monomorphic for each gene. Our data confirm that the snails limit their movements to an area with a radius of a few meters, and that they show active homing when taken outside this area (Mrs. G. Ballintijn-de Vries and L. de Ruiter, unpublished). The conclusion seems inevitable that selection itself must make for polymorphism rather than uniformity of colonies by favouring intermediate and not extreme allele frequencies. Further investigations on this point would be very valuable.

Even though we may never be able to give a completely satisfactory account of the contributions of selection and sampling errors in bringing about the present genetic composition of even a single *Cepaea* colony, this at least seems clear that the intensity of selection in natural populations of these snails may be very high.

References:

Boettger, C. R., 1931: Z. ind. Abst. Vererb., **58,** 295–316.
Boettger, C. R., 1954: Biol. Zentr. bl., **73,** 318–333.
Cain, A. and P. M. Sheppard, 1954: Genetics, **39,** 89–116.
Diver, C., 1940: In: The new systematics, J. Huxley editor, Oxford, 303–328.

4. A. D. Voûte (Arnhem). Outbreaks and regulation of the population-density of *Ascaris lumbricoides* L. in the Netherlands.

In some parts of the Netherlands the population is heavily infected with *Ascaris lumbricoides* L.. The epidemiology of this species was investigated in cooperation with Dr. J. A. Troelstra of the Paediatric Clinic of the University Hospital at Groningen.

The influence of temperature on the development of the eggs was investigated. At 30°C the eggs died soon after maturation. Optimum development took place at ± 20°C.

In biologically active soils (clay, leaf mould etc.) mortality among mature eggs, i.c. containing motile larvae, was very high. In the kitchen gardens at Tange Alteveer, manured with heavily infected night soil, the egg disappeared from the soil within 3 months.

On vegetables from these gardens no eggs could be found.

Measurements of pig-ascarids, collected in the abattoir at Arnhem proved the existence of a factor causing mortality among the larvae migrating in the body of the host which factor increased in intensity as long as the larvae migrated. Also, the presence of adult *Ascaris* in the

intestine of the host appeared to prevent the development of the larvae or the hatching of the eggs. Both factors probably cooperate in regulating the population-density of the ascarids in the host.

The height of the level at which that population is regulated is also influenced by the number of eggs in the source of infection and in the intensity of contact with that source.

Heavy infections have been observed in families living in poor hygienic conditions but only if they manured the kitchen gardens with night soil. Hygienically living families, using night soil in their gardens were not or only slightly infected. The source of infection appeared to be the privy.

5. J. W. Sluiter (Utrecht). Distribution and population size of bats hibernating in S. Limburg, 1942–1957.

Voir: Sluiter and Van Heerdt, Publ. Nat. Hist. Genootsch. Limburg, in the press.